US009422582B2

(12) **United States Patent**
Anthony et al.

(10) **Patent No.: US 9,422,582 B2**
(45) **Date of Patent: *Aug. 23, 2016**

(54) **HOST CELLS AND METHODS FOR PRODUCTION OF ISOBUTANOL**

(71) Applicant: **BUTAMAX(TM) ADVANCED BIOFUELS LLC**, Wilmington, DE (US)

(72) Inventors: **Larry Cameron Anthony**, Aston, PA (US); **Hongxian He**, Wilmington, DE (US); **Lixuan Lisa Huang**, Hockessin, DE (US); **Daniel P. Okeefe**, Ridley Park, PA (US); **Arthur Leo Kruckeberg**, Wilmington, DE (US); **Yougen Li**, Pennington, NJ (US); **Lori Ann Maggio-Hall**, Wilmington, DE (US); **Jessica McElvain**, Wilmington, DE (US); **Mark J. Nelson**, Newark, DE (US); **Ranjan Patnaik**, Newark, DE (US); **Steven Cary Rothman**, Wilmington, DE (US)

(73) Assignee: **Butamax Advanced Biofuels LLC**, Wilmington, DE (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **13/840,676**

(22) Filed: **Mar. 15, 2013**

(65) **Prior Publication Data**

US 2014/0030783 A1 Jan. 30, 2014

Related U.S. Application Data

(63) Continuation of application No. 13/428,585, filed on Mar. 23, 2012.

(60) Provisional application No. 61/472,484, filed on Apr. 6, 2011, provisional application No. 61/467,261, filed on Mar. 24, 2011, provisional application No. 61/472,487, filed on Apr. 6, 2011, provisional application No. 61/467,271, filed on Mar. 24, 2011, provisional application No. 61/570,513, filed on Dec. 14, 2011, provisional application No. 61/467,249, filed on Mar. 24, 2011, provisional application No. 61/472,497, filed on Apr. 6, 2011, provisional application No. 61/472,474, filed on Apr. 6, 2011.

(51) **Int. Cl.**

C12P 7/16	(2006.01)
C12N 15/81	(2006.01)
C12N 9/04	(2006.01)
C12P 7/42	(2006.01)
C12N 1/38	(2006.01)

(52) **U.S. Cl.**

CPC ... ***C12P 7/16*** (2013.01); ***C12N 1/38*** (2013.01); ***C12N 9/0006*** (2013.01); ***C12N 15/81*** (2013.01); ***C12N 15/815*** (2013.01); ***C12P 7/42*** (2013.01); *Y02E 50/10* (2013.01); *Y02P 20/52* (2015.11)

(58) **Field of Classification Search**

CPC C12P 7/16; C12P 7/42; C12N 15/81; C12Y 101/01086; C12Y 202/01006

USPC 435/160

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,683,202	A	7/1987	Mullis et al.	
4,865,973	A *	9/1989	Kollerup et al.	435/43
5,643,779	A	7/1997	Ehrlich et al.	
6,586,229	B1	7/2003	Ben-Bassat et al.	
6,960,465	B1	11/2005	Papoutsakis et al.	
7,541,173	B2	6/2009	Bramucci et al.	
7,659,104	B2	2/2010	Bramucci et al.	
7,851,188	B2	12/2010	Donaldson et al.	
7,910,342	B2	3/2011	Liao et al.	
7,993,889	B1	8/2011	Donaldson et al.	
8,017,364	B2	9/2011	Bramucci et al.	
8,017,376	B2	9/2011	Dundon et al.	
8,043,638	B2	10/2011	Omura	
8,071,358	B1 *	12/2011	Dundon et al.	435/254.2
8,097,440	B1	1/2012	Buelter et al.	
8,101,808	B2	1/2012	Evanko et al.	
8,129,162	B2	3/2012	Li et al.	
8,133,715	B2	3/2012	Buelter et al.	
8,153,415	B2	4/2012	Buelter et al.	
8,158,404	B2	4/2012	Lies et al.	
8,178,328	B2	5/2012	Donaldson et al.	

(Continued)

FOREIGN PATENT DOCUMENTS

WO	WO9408020	4/1994
WO	0061722 A1	10/2000

(Continued)

OTHER PUBLICATIONS

Davison BH et al. Continuous Direct Solvent Extration of Butanol in a Fermenting Fluidized-Bed Bioreactor with Immobilized Clostridium acetobutylicum. 1993. Applied Biochemistry and Biotechnology. vol. 39/40. p. 415-426.*

Kumenovics A et al. Identification of FRA1 and FRA2 as genes involved in regulating the yeast iron regulon in response to decreased mitochondrial iron-sulfur cluster synthesis. 2008. Journal of Biological Chemistry. 283:10276-10286.*

GenBank EDR97797.1. Feb. 12, 2008. 1 page.*

Abbad-Andaloussi, et al., Carbon and Electron Flow in Clostridium butyricum grown in Chemostat Culture on Glycerol and on Glucose, Microbiology 142:1149-1158, 1996.

(Continued)

Primary Examiner — Paul Holland

(57) **ABSTRACT**

Provided herein are recombinant yeast host cells and methods for their use for production of isobutanol. Yeast host cells provided comprise an isobutanol biosynthetic pathway and at least one of reduced or eliminated aldehyde dehydrogenase activity, reduced or eliminated acetolactate reductase activity; or a heterologous polynucleotide encoding a polypeptide having ketol-acid reductoisomerase activity.

16 Claims, 34 Drawing Sheets

(56) **References Cited**

U.S. PATENT DOCUMENTS

8,188,250	B2	5/2012	Bramucci et al.	
8,206,970	B2	6/2012	Eliot et al.	
8,222,017	B2	7/2012	Li et al.	
8,232,089	B2	7/2012	Urano et al.	
8,241,878	B2	8/2012	Anthony et al.	
8,273,558	B2	9/2012	Donaldson et al.	
8,273,565	B2	9/2012	Dundon et al.	
8,283,144	B2	10/2012	Donaldson et al.	
8,372,612	B2	2/2013	Larossa et al.	
8,389,252	B2	3/2013	Larossa	
8,455,224	B2	6/2013	Paul	
8,455,225	B2	6/2013	Bramucci et al.	
8,455,239	B2	6/2013	Feldman et al.	
8,465,964	B2	6/2013	Anthony	
8,518,678	B2	8/2013	Flint et al.	
8,557,562	B2	10/2013	Bramucci et al.	
8,614,085	B2	12/2013	Van Dyk	
8,637,281	B2	1/2014	Paul et al.	
8,637,289	B2	1/2014	Anthony et al.	
8,652,823	B2	2/2014	Flint et al.	
8,669,094	B2	3/2014	Anthony et al.	
8,691,540	B2	4/2014	Bramucci et al.	
8,735,114	B2	5/2014	Donaldson et al.	
8,765,433	B2	7/2014	Gude et al.	
8,785,166	B2	7/2014	Anthony	
8,795,992	B2	8/2014	Bramucci et al.	
8,828,694	B2	9/2014	Anthony et al.	
8,828,704	B2	9/2014	Donaldson et al.	
8,871,488	B2	10/2014	Dauner et al.	
8,889,385	B2	11/2014	Donaldson et al.	
8,895,307	B2	11/2014	Li et al.	
8,906,666	B2	12/2014	Alsaker et al.	
8,911,981	B2	12/2014	Li et al.	
8,940,511	B2	1/2015	Larossa	
8,945,859	B2	2/2015	Donaldson et al.	
8,945,899	B2	2/2015	Li et al.	
8,951,774	B2	2/2015	Donaldson	
8,951,937	B2	2/2015	Flint et al.	
8,956,850	B2	2/2015	Anthony	
8,962,298	B2	2/2015	Donaldson et al.	
8,969,065	B2	3/2015	Anthony et al.	
8,980,612	B2	3/2015	Donaldson et al.	
2002/0061569	A1	5/2002	Haselbeck et al.	
2004/0234649	A1	11/2004	Lewis et al.	
2004/0248250	A1	12/2004	Nakai et al.	
2005/0059136	A1	3/2005	van Maris et al.	
2005/0112739	A1	5/2005	Golubkov et al.	
2007/0031918	A1	2/2007	Dunson, Jr. et al.	
2007/0031950	A1	2/2007	Winkler	
2007/0092957	A1	4/2007	Donaldson et al.	
2007/0259411	A1	11/2007	Bramucci et al.	
2008/0182308	A1	7/2008	Donaldson et al.	
2008/0261230	A1	10/2008	Liao et al.	
2008/0261861	A1	10/2008	Sleep et al.	
2009/0081746	A1	3/2009	Liao et al.	
2009/0163376	A1*	6/2009	Li et al.	506/9
2009/0269823	A1	10/2009	Bramucci et al.	
2009/0305363	A1	12/2009	Anthony et al.	
2010/0081154	A1	4/2010	Flint et al.	
2010/0081173	A1	4/2010	Park et al.	
2010/0081179	A1	4/2010	Anthony et al.	
2010/0081182	A1	4/2010	Paul et al.	
2010/0081183	A1	4/2010	Paul et al.	
2010/0093020	A1	4/2010	Bramucci et al.	
2010/0112655	A1	5/2010	Paul et al.	
2010/0120105	A1	5/2010	Anthony et al.	
2010/0124773	A1	5/2010	Yang et al.	
2010/0129887	A1	5/2010	Anthony et al.	
2010/0143997	A1	6/2010	Buelter et al.	
2010/0197519	A1	8/2010	Li et al.	
2011/0076733	A1*	3/2011	Urano et al.	435/160
2011/0111472	A1	5/2011	Donaldson et al.	
2011/0124060	A1	5/2011	Anthony et al.	
2011/0136192	A1	6/2011	Paul et al.	
2011/0195505	A1	8/2011	Euler et al.	
2011/0201072	A1	8/2011	Bastian et al.	
2011/0201073	A1*	8/2011	Buelter	C12N 9/0006 435/160
2011/0244536	A1	10/2011	Nagarajan et al.	
2011/0250610	A1	10/2011	Bramucci et al.	
2011/0275129	A1	11/2011	Buelter et al.	
2012/0058541	A1	3/2012	Alsaker et al.	
2012/0064561	A1	3/2012	Flint et al.	
2012/0149080	A1	6/2012	Bramucci et al.	
2012/0196341	A1	8/2012	Donaldson et al.	
2012/0237988	A1	9/2012	Anthony et al.	
2012/0258873	A1	10/2012	Gibson et al.	
2013/0035515	A1	2/2013	Dobson et al.	
2013/0071898	A1	3/2013	Anthony et al.	
2013/0171706	A1	7/2013	Donaldson et al.	
2013/0203138	A1	8/2013	McElvain et al.	
2013/0252296	A1	9/2013	Maggio-Hall	
2013/0316414	A1	11/2013	Paul	
2013/0344551	A1	12/2013	Li et al.	
2014/0004526	A1	1/2014	Dauner et al.	
2014/0030782	A1	1/2014	Anthony et al.	
2014/0030783	A1	1/2014	Anthony et al.	
2014/0038263	A1	2/2014	Flint et al.	
2014/0038268	A1	2/2014	Flint et al.	
2014/0051133	A1	2/2014	Govindarajan et al.	
2014/0051137	A1	2/2014	Flint et al.	
2014/0057329	A1	2/2014	Li et al.	
2014/0093930	A1	4/2014	Li et al.	
2014/0096439	A1	4/2014	Bramucci et al.	
2014/0141479	A1	5/2014	Anthony et al.	
2014/0170732	A1	6/2014	Bramucci et al.	
2014/0186910	A1	7/2014	Rothman et al.	
2014/0186911	A1	7/2014	Anthony et al.	
2014/0273116	A1	9/2014	Kelly et al.	
2014/0273129	A1	9/2014	Bhalla et al.	
2014/0308735	A1	10/2014	Anthony	
2014/0335582	A1	11/2014	Donaldson et al.	
2014/0349349	A1	11/2014	Dauner et al.	
2014/0377824	A1	12/2014	Satagopan et al.	
2015/0037855	A1	2/2015	Bhadra et al.	
2015/0111269	A1	4/2015	Li et al.	
2015/0119608	A1	4/2015	Donaldson et al.	
2015/0125920	A1	5/2015	Anthony et al.	

FOREIGN PATENT DOCUMENTS

WO	WO0112833		2/2001
WO	WO2005040392		5/2005
WO	WO2008098227		8/2008
WO	WO2008130995		10/2008
WO	WO2009056984		5/2009
WO	WO 2009056984	A1 *	5/2009
WO	WO2009059253		5/2009
WO	WO2009078108		6/2009
WO	WO2009086423		7/2009
WO	WO 2010051527	A2 *	5/2010
WO	WO2010062597		6/2010
WO	WO2011019894		2/2011
WO	2012027642	A1	3/2012

OTHER PUBLICATIONS

Arthur, et al., Contribution of VanY D,D-Carboxypeptidase to Glycopeptide Resistance in Enterococcus faecalis by Hydrolysis of Peptidoglycan Precursors, Antimicrob. Agents Chemother. 38:1899-1903, 1994.

Aulabaugh, et al., Oxalyl Hydroxamates as Reaction-Intermediate Analogues for Ketol-Acid Reductoisomerase, Biochemistry 29:2824-2830 1990.

Biou, et al. The crystal structure of plant acetohydroxy acid isomeroreductase complexed with NADPH, two magnesium ions and a herbicidal transition state analog determined at 1.65 Å resolution, EMBO Journal 16:3405-3415, 1997.

van der Geize, et al., Targeted Disruption of the kstD Gene Encoding a 3-Ketosteroid delta1-Dehydrogenase Isoenzyme of Rhodococcus erythropolis Strain SQ1, Appl. Environ. Microbiol. 66:2029-2036, 2000.

(56) **References Cited**

OTHER PUBLICATIONS

Chunduru, et al., Mechanism of Ketol Acid Reductoisomerase—Steady State Analysis and Metal Ion Requirement, Biochemistry 28:486-493 1989.

de Cavalho, et al., *Mycobacterium* sp., Rhodococcus erythropolis, and Pseudomonas putida Behavior in the Presence of Organic Solvents, Microsc. Res. Tech. 64:215-22, 2004.

de la Plaza, et al., Biochemical and molecular characterization of alpha-ketoisovalerate decarboxylase, an enzyme involved in the formation of aldehydes from amino acids by Lactococcus lactis, FEMS Microbiol. Lett. 238:367-374, 2004.

Deshpande, Ethanol Production from Cellulose by Coupled Saccharification/Fermentation using *Saccharomyces cerevisiae* and Cellulase Complex from Sclerotium rolfsii UV-8 Mutant, Appl. Biochem. Biotechnol. 36:227, 1992.

Dürre, et al., Solventogenic Enzymes of Clostridium acetobutylicum: Catalytic Properties, Genetic Organization, and Transcriptional Regulation, FEMS Microbiol. Rev. 17:251-262, 1995.

Dürre, New insights and novel developments in clostridial acetone/butanol/isopropanol fermentationAppl. Microbiol. Biotechnol. 49:639-648, 1998.

Eichenbaum, et al., Use of the Lactococcal nisA Promoter to Regulate Gene Expression in Gram-Positive Bacteria: Comparison of Induction Level and Promoter Strength, Appl. Environ. Microbiol. 64:2763-2769, 1998.

Fleming, et al., Extracellular Enzyme Synthesis in a Sporulation-Deficient Strain of Bacillus licheniformis, Appl. Environ. Microbiol. 61:3775-3780, 1995.

Flint, et al., The Role and Properties of the Iron-Sulfur Cluster in *Escherichia coli* Dihydroxy-acid Dehydratase, J. Biol. Chem. 268:14732-14742, 1993.

Ford, et al., Characterization of Ypr1p from *Saccharomyces cerevisiae* as a 2-methylbutyraldehyde reductase, Yeast 19:1087-1096, 2002.

Fujimoto, et al., pAM401-Based Shuttle Vectors That Enable Overexpression of Promoterless Genes and One-Step Purification of Tag Fusion Proteins Directly from Enterococcus faecalis, Appl. Environ. Microbiol. 67:1262-1267, 2001.

Gollop, et al., Physiological Implications of the Substrate Specificities of Acetohydroxy Acid Synthases from Varied Organisms, J. Bacteriol. 172:3444-3449, 1990.

Groot, et al., Technologies for Butanol Recovery Integrated with Fermentations, Process. Biochem. 27:61-75, 1992.

Guex, et al., SWISS-MODEL and the Swiss-PdbViewer: An Environment for Comparative Protein Modeling, Electrophoresis 18:2714-2723, 1997.

Hermann, et al., Isolation and Characterization of Butanol-Resistant Mutants of Clostridium acetobutylicum, Appl. Environ. Microbiol. 50:1238-1243, 1985.

Holtzclaw, et al., Degradative Acetolactate Synthase of Bacillus subtilis: Purification and Properties, J. Bacteriol. 121:917-922, 1975.

Jones, et al., Improved Methods for Building Protein Models in Electron Density Maps and the Location of Errors in these Models, Acta Crystallogr. A 47:110-119, 1991.

Kabelitz, et al., Effect of aliphatic alcohols on growth and degree of saturation of membrane lipids in Acinetobacter calcoaceticus, FEMS Microbiol. Lett. 220: 223-227, 2003.

Datsenko, et al., One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products, Proc. Natl. Acad. Sci. USA 97:6640-6645, 2000.

Kleerebezem, et al., Controlled Gene Expression Systems for Lactic Acid Bacteria: Transferable Nisin-Inducible Expression Cassettes for *Lactococcus, Leuconostoc*, and *Lactobacillus* spp., Appl. Environ. Microbiol. 63:4581-4584, 1997.

Kostichka, et al., A small cryptic plasmid from Rhodococcus erythropolis : characterization and utility for gene expressionAppl. Microbiol. Biotechnol. 62:61-68, 2003.

Krogh, et al., Hidden Markov Models in Computational Biology, J. Mol. Biol. 235:1501-1531, 1994.

Larroy, et al., Characterization of the *Saccharomyces cerevisiae* YMR318C (ADH6) gene product as a broad specificity NADPH-dependent alcohol dehydrogenase: relevance in aldehyde reduction, Biochem. J. 361:163-172, 2002.

Maguin, et al., New Termosensitive Plasmid for Gram-Positive Bacteria, J. Bacteriol. 174:5633-5638, 1992.

Marinus, et al., Regulation of Isoleucine-Valine Biosynthesis in *Pseudomonas aeruginosa*, Genetics 63:547-56, 1969.

Nagarajan, et al., Modular Expression and Secretion Vectors for Bacillus subtilis, Gene 114:121-126, 1992.

Nakashima, et al., Isolation and Characterization of a Rolling-Circle-Type Plasmid from Rhodococcus erythropolis and Application of the Plasmid to Multiple-Recombinant-Protein Expression, Appl. Environ. Microbiol. 70:5557-5568, 2004.

Nallaapareddy, et al., Construction of Improved Temperature-Sensitive and Mobilizable Vectors and Their Use for Constructing Mutations in the Adhesin-Encoding acm Gene of Poorly Transformable Clinical Enterococcus faecium Strains, Appl. Environ. Microbiol. 72:334-345, 2006.

O'Sullivan, et al., High- and low-copy-number Lactococcus shuttle cloning vectors with features for clone screening, Gene 137:227-231, 1993.

Payne, et al., Use of Alkaline Phosphatase Fusions to Study Protein Secretion in Bacillus subtilis, J. Bacteriol. 173:2278-2282, 1991.

Renault, et al., Plasmid Vectors for Gram-positive Bacteria Swithching from High to Low Copy Number, Gene 183:175-182, 1996.

Scott, et al., Sequences of versatile broad-host-range vectors of the RK2 family, Plasmid 50:74-79, 2003.

Smit, et al., Identification, Cloning, and Characterization of a Lactococcus lactis Branched-Chain alpha-Keto Acid Decarboxylase Involved in Flavor Formation, Appl. Environ. Microbiol. 71:303-311, 2005.

Sulter, et al., Proliferation and metabolic significance of peroxisomes in Candida boidinii during drowth on D-alanine or oleic acid as the sole carbon source, Arch. Microbiol. 153:485-489, 1990.

Sulzenbacher, et al., Crystal Structure of *E. coli* Alcohol Dehydrogenase YqhD: Evidence of a Covalently Modified NADP Coenzyme, J. Mol. Biol. 342:489-502, 2004.

Tabor, et al., A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes, Proc. Acad. Sci. USA 82:1074, 1985.

Taghavi, et al., Electroporation of Alcaligenes eutrophus with (Mega) Plasmids and Genomic DNA Fragments, Appl. Environ. Microbiol. 60:3585-3591, 1994.

Tanimoto, et al., Analysis of the Conjugal Transfer System of the Pheromone-Independent Highly Transferable Enterococcus Plasmid pMG1: Identification of a tra Gene (traA) Up-Regulated during Conjugation, J. Bacteriol. 184:5800-5804, 2002.

Tao, et al., Construction of highly efficient *E. coli* expression systems containing low oxygen induced promoter and partition region, Appl. Microbiol. Biotechnol. 68:346-354, 2005.

Thompson, et al., CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nuc. Acid Res. 22:4673-4680, 1994.

Tomas, et al., Transcriptional Analysis of Butanol Stress and Tolerance in Clostridium acetobutylicum, J. Bacteriol. 186:2006-2018, 2004.

Tyagi, et al., The crystal structure of a bacterial Class II ketol-acid reductoisomerase: Domain conservation and evolution, Protein Sci. 14:3089-3100, 2005.

van Kranenburg, et al., Functional Analysis of Three Plasmids from Lactobacillus plantarum, Appl. Environ. Microbiol. 71:1223-1230, 2005.

Walker, et al., Isothermal in vitro amplification of DNA by a restriction enzyme/DNA polymerase systemProc. Natl. Acad. Sci. U.S.A. 89:392-396, 1992.

Wyckoff, et al., Characterization and Sequence Analysis of a Stable Cryptic Plasmid from Enterococcus faecium 226 and Development of a Stable Cloning Vector, Appl. Environ. Microbiol. 62:1481-1486, 1996.

(56) **References Cited**

OTHER PUBLICATIONS

Dumas, et al., Purification and characterization of a fusion protein of plant acetohydroxy acid synthase and acetohydroxy acid isomeroreductase, FEBS Lett. 408:156-160, 1997.

Bellion et al., Microb. Growth C1 Compd., [Int. Symp.], 7th (1993), 415 32. (eds): Murrell, J. Collin; Kelly, Don P. Publisher: Intercept, Andover, UK.

Dumas, et al., Isolation and kinetic properties of acetohydroxy acid isomeroreductase from spinach (*Spinacia oleracea*) chloroplasts overexpressed in *Escherichia coli*, Biochem. J. 288:865-874, 1992.

Epelbaum, et al. Branched-chain amino acid biosynthesis in *Salmonella typhimurium*: a quantitative analysis, J. Bacteriol. 180:4056-4067, 1998.

Kuzuyama, Mevalonate and nonmevalonate pathways for the biosynthese of isoprene units, Biosci. Biotechnol. Biochem. 66:1619-1627, 2002.

Garcia, et al. Fusel alcohols production in beer fermentation processes, Proc. Biochem. 29:303-309,1994.

Carlini, et al., Guerbet condensation of methanol with n-propanol to isobutyl alcohol over heterogeneous copper chromite/Mg—Al mixed oxides, J. Mol. Catalysis A 220:215-220, 2004.

Rothstein, Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast, Meth. Enzymol. 194:281-301, 1991.

Horton, et al., Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension, Gene 77:61-68, 1989.

Kaneko, et al., Complete Genome Sequence of the Filamentous Nitrogen-fixing Cyanobacterium *Anabaena* sp. strain PCC 7120, DNA Res. 8:205-213, 227-253, 2001.

Dickinson, et al., An Investigation of the Metabolism to Isobutyl Alcohol in *Saccharomyces cerevisiae*, J. Biol. Chem. 273:25752-25756, 1998.

Oaxaca, et al., Formation of Ethanol and Higher Alcohols by Immobilized Zymomonas mobilis in Continuous Culture, ACTA Biotechnol. 11:523-532, 1991.

Eppink, et al., Switch of Coenzyme Specificity of p-Hydroxybenzoate Hydroxylase, J. Mol. Biol. 292:87-96, 1999.

Nakanishi, et al., Switch of Coenzyme Specificity of Mouse Lung Carbonyl Reductase by Substitution of Threonine 38 with Aspartic Acid, J. Biol. Chem. 272:2218-2222, 1997.

Kamerbeek, et al., Identifying Determinants of NADPH Specificity in Baeyer-Villiger Monooxygenases, Eur. J. Biochem. 271:2107-2116, 2004.

Nishiyama, et al., Alteration of Coenzyme Specificity of Malate Dehydrogenase from Thermus flavus by Site-directed Mutagenesis, J. Biol. Chem. 268:4656-4660, 1993.

Martinez-Julvez, et al., Towards a New Interaction Enzyme: Coenzyme, Biophys. Chem. 115:219-224, 2005.

Rane, et al., Reversal of the Nucleotide Specificity of Ketol Acid Reductoisomerase by Site-Directed Mutagenesis Identifies the NADPH Binding Site, Archiv. Biochem. Biophys. 338:83-89, 1997.

Ahn, et al., Crystal Structure of Class I Acetohydroxy Acid Isomeroreductase from Pseudomonas aeruginosa, J. Mol. Biol. 328:505-515, 2003.

Paulsen, et al., Complete genome sequence of the plant commensal Pseudomonas fluorescens Pf-5, Nature Biotechnol. 23:8730878, 2005.

Carugo, et al., NADP-Dependent Enzymes I: Conserved Stereochemistry of Cofactor Binding, Proteins: Structure, Function, and Genetics 28:10-28, 1997.

Dumas, et al., Enzymology, Structure, and Dynamics of Acetohydroxy Acid Isomeroreductase, Acc. Chem. Res. 34:399-408, 2001.

Elmore, et al., Modification of the Nucleotide Cofactor-binding Site of Cytochrome P-450 Reductase to Enhance Turnover with NADH in vivo, J. Biol. Chem. 277:48960-48964, 2002.

Fisher, et al., The X-ray Structure of Brassica napus beta-keto acyl carrier protein reductase and its implication for substrate binding and catalysis, Structure 8:339-347, 2000.

Khoury, et al., Computational design of Candida boidinii xylose reductase for altered cofactor specificity, Protein Sci. 18:2125-2136, 2009.

Kuzuyama, et al., Characterization of 1-deoxy-D-xylulose 5-Phosphate Reductoisomerase, an Enzyme Involved in Isopentenyl Diphosphate Biosynthesis, and Identification of Its Catalytic Amino Acid Residues, J. Biol. Chem. 275:19928-19932, 2000.

Medina, et al., Probing the Determinants of Coenzyme Specificity in Ferredoxin-NADP+ Reductase by Site-directed Mutagenesis, J. Biol. Chem. 276:11902-11912, 2001.

Wierenga, et al., Predication of the Occurence of the SDP-binding Beta-alpha-beta Fold in Proteins, Using an Amino Acid Sequence Fingerprint, J. Mol. Biol. 187:101-107, 1986.

Brinkmann-Chen, et al., General approach to reversing ketol-acid reductoisomerase cofactor dependence from NADPH to NADH, Proc. Natl. Acad. Sci. 110:10946-10951, 2013.

Rossmann, et al., Chemical and biological evolution of nucleotide-binding protein, Nature 250:194-199, 1974.

Curien, et al., Nucleotide sequence and characterization of a cDNA encoding the acetohydroxy acid isomeroreductase from Arabidopsis thaliana, Plant Molecular Biology 21:717-722, 1993.

Dumas, et al., Purification and characterization of acetohydroxyacid reductoisomerase from spinach chloroplasts, Biochem. J. 262:971-976, 1989.

Durner, et al., Ketol-Acid Reductoisomerase from Barley (*Hordeum vulgare*): Purification. Properties, and Specific Inhibition, Plant Physiol. 103:903-910, 1993.

Feeney, et al., A single amino acid substitution in lactate dehydrogenase improves the catalytic efficiency with an alternative coenzyme, Biochem. Biophys. Res. Commun. 166:667-672, 1990.

Inui, et al., Identification and sequence determination of the acetohydroxy acid isomeroreductase gene from Brevibacterium flavum MJ233, DNA Seq. 4:95-103, 1993 (Abstract).

Lauvergeat, et al., Site-directed mutagenesis of a serine residue in cinnamyl alcohol dehydrogenase, a plant NADPH-dependent dehydrogenase, affects the specificity for the coenzyme, Biochemistry 34: 12426-12434, 1995.

Levskaya, et al., Synthetic biology: engineering *Escherichia coli* to see light, Nature 438:441-442, 2005.

Shiraishi, et al., Engineering of pyridine nucleotide specificity of nitrate reductase: mutagenesis of recombinant cytochrome b reductase fragment of Neurospora crassa NADPH:Nitrate reductase, Archives of Biochemistry and Biophysics 358:104-115, 1998.

Tyagi, et al., Probing the mechanism of the bifunctional enzyme ketol-acid reducoisomerase by site-directed mutagenesis of the active site, FEBS Journal 272:593-602, 2005.

Zhang, et al., Change of nucleotide specificity and enhancement of catalytic efficiency in single point mutants of Vibrio harveyi aldehyde dehydrogenase, Biochemistry 38:11440-11447, 1999.

International Preliminary Report on Patentability date Sep. 24, 2013 for International Application No. PCT/US2012/030479.

U.S. Appl. No. 13/428,585, filed Mar. 23, 2012.

EBI Accession No. UniProt: Q8ZAC2, Entry Date Jun. 6, 2003.

EBI Accession No. UniProt: Q0AV19, Entry Date Jan. 15, 2008.

EBI Accession No. UniProt: Q02138, Entry Date Jul. 1, 1993.

EBI Accession No. UniProt: Q01292, Entry Date Apr. 1, 1993.

EBI Accession No. UniProt: P06168, Entry Date Jan. 1, 1988.

EBI Accession No. UniProt: P05793, Entry Date Nov. 1, 1988.

EBI Accession No. UniProt: B1ZV88, Entry Date Jun. 6, 2003.

She, et al., Q97YJ9—UNIPROTKB/Swiss-Prot. Database, Oct. 31, 2006.

Suerbaum, et al., UniProtKB Database, Accession Q7VGW6, 2003.

Kaneko, et al., Q8YUM—UniProt Database, Mar. 23, 2010.

GenBank No. NC_009135.1, Methanococcus maripaludis C5, complete genome, Apr. 30, 2009.

GenBank No. NC_005791.1, Methanococcus maripaludis S2, complete genome, Apr. 25, 2009.

GenBank No. NZ_AAWX01000002.1, Copeland, et al., Feb. 7, 2007; pp. 1-3.

GenBank No. NC_001144.4, *Saccharomyces cerevisiae* chromosome XII, complete sequence, Jun. 16, 2008.

GenBank No. NC_002754.1, Sulfolobus solfataricus P2, complete genome, Apr. 26, 2009.

(56) **References Cited**

OTHER PUBLICATIONS

GenBank No. NC_003364.1, Pyrobaculum aerophilum str. IM2, complete genome, Apr. 24, 2009.
GenBank No. AAA25079, acetolactate synthase [Klebsiella pneumoniae], Aug. 5, 1994.
GenBank No. AAA25161, alpha-acetolactate synthase, Apr. 21, 1994.
GenBank No. AAA65614, keto acid dehydrogenase E1-alpha subunit [Pseudomonas putida] Feb. 27, 2002.
GenBank No. AAA65615, 39 kDa keto acid dehydrogenase E1-beta subunit [Pseudomonas putida], Feb. 27, 2002.
GenBank No. AAA65617, transacylase E2 [Pseudomonas putida], Feb. 27, 2002.
GenBank No. AAA65618, lipoamide dehydrogenase [Pseudomonas putida], Feb. 27, 2002.
GenBank No. AAS49166, branched-chain alpha-ketoacid decarboxylase [Lactococcus lactis], Dec. 27, 2004.
GenBank No. AJ746364, *Lactococcus lactis* subsp. lactis kivd gene for alpha-ketoisovalerate decarboxylase, strain IFPL730, Apr. 15, 2005.
GenBank No. AY548760, Lactococcus lactis branched-chain alpha-ketoacid decarboxylase (kdcA) gene, complete cds. Dec. 27, 2004.
GenBank No. BX950229, Methanococcus maripaludis strain S2, complete sequence, May 8, 2008.
GenBank No. CAB14105, dihydroxy-acid dehydratase [*Bacillus subtilis* subsp. *subtilis* str, Oct. 1, 2009.
GenBank No. CAB14334, branched-chain alpha-keto acid dehydrogenase E2 subunit (lipoamide acyltransferase) [*Bacillus subtilis* subsp. *subtilis* str. 168], Oct. 1, 2009.
GenBank No. CAB14335, branched-chain alpha-keto acid dehydrogenase E1 subunit [*Bacillus subtilis* subsp. *subtilis* str. 168], Oct. 1, 2009.
GenBank No. CAB14336, branched-chain alpha-keto acid dehydrogenase E1 subunit [*Bacillus subtilis* subsp. *subtilis* str. 168], Oct. 1, 2009.
GenBank No. CAB14337, branched-chain alpha-keto acid dehydrogenase E3 subunit (dihydrolipoamide dehydrogenase) [*Bacillus subtilis* subsp. *subtilis* str. 168], Oct. 1, 2009.
GenBank No. CAB15618, alpha-acetolactate synthase [*Bacillus subtilis* subsp. *subtilis* str. 168], Oct. 1, 2009.
GenBank No. CAF29874, Dihydroxy-acid dehydratase [Methanococcus maripaludis S2], May 8, 2008.
GenBank No. CAG34226, alpha-ketoisovalerate decarboxylase [*Lactococcus lactis* subsp. *lactis*], Apr. 15, 2005.
GenBank No. L16975, Lactococcus lactis alpha-acetolactate synthase (als) gene, complete cds, Apr. 21, 1994.
GenBank No. M57613, Pseudomonas putida branched-chain keto acid dehydrogenase operon (bkdA1, bkdA1 and bkdA2), transacylase E2 (bkdB), bkdR and lipoamide dehydrogenase (lpdV) genes, complete cds, Feb. 27, 2002.
GenBank No. M73842, Klebsiella pneumoniae acetolactate synthase (iluk) gene, complete cds, Aug. 5, 1994.
GenBank No. NC_001142, Nosema ceranae BRL01 Nc001142, whole genome shotgun sequence, Jun. 9, 2009.
GenBank No. NC_003030, Clostridium acetobutylicum ATCC 824, complete genome, Oct. 22, 2009.
GenBank No. NC_001136, *Saccharomyces cerevisiae* chromosome IV, complete sequence, Dec. 9, 2009.
GenBank No. NC_001145, *Saccharomyces cerevisiae* chromosome XIII, complete sequence, Dec. 9, 2009.
GenBank No. NC_001988, Clostridium acetobutylicum ATCC 824 plasmid pSOL1, complete sequence, Apr. 26, 2009.
GenBank No. NC_003197, *Salmonella typhimurium* LT2, complete genome, Mar. 30, 2010.
GenBank No. NP_012550, Dihydroxyacid dehydratase, catalyzes third step in the common pathway leading to biosynthesis of branchedchain amino acids; llv3p [*Saccharomyces cerevisiae*], Nov. 5, 2009.
GenBank No. NP_010656, Jacq, et al., downloaded Apr. 15, 2010, pp. 1-3.
GenBank No. NP_014051, Adh6p [*Saccharomyces cerevisiae*], Dec. 9, 2009.
GenBank No. NP_149189, pyruvate decarboxylase [Clostridium acetobutylicum ATCC 824], Apr. 26, 2009.
GenBank No. NP_349892, NADH-dependent butanol dehydrogenase A (BDH I) [Clostridium acetobutylicum ATCC 824], Apr. 14, 2010.
GenBank No. NP_417484, alcohol dehydrogenase, NAD(P)-dependent [*Escherichia coli* str. K-12 substr. MG1655]. Apr. 9, 2010.
GenBank No. NP_461346, indolepyruvate decarboxylase [*Salmonella typhimurium* LT2], Apr. 30, 2009.
GenBank No. YP_026248, dihydroxyacid dehydratase [*Escherichia coli* str. K-12 substr. MG1655], Jul. 30, 2009.
GenBank No. Z99115, Bacillus subtilis complete genome (section 12 of 21): from 2207806 to 2409180, Nov. 15, 2007.
GenBank No. AL009126, *Bacillus subtilis* subsp. *subtilis* str. 168 complete genome, Oct. 1, 2009.
GenBank No. Z99122, *Bacillus subtilis* complete genome (section 19 of 21): from 3608981 to 3809670, Apr. 18, 2005.
GenBank No. ZP01224863.1, ketol-acid reductoisomerase [marine gamma proteobacterium HTCC2207], Mar. 24, 2006.
GenBank No. NC_003295.1, Ralstonia solanacearum GMI1000, complete genome, May 1, 2009.
GenBank No. NC_002516, Pseudomonas aeruginosa PAO1, Jul. 20, 2008.
GenBank No. NC_004129, Pseudomonas fluorescens Pf-5, Jul. 20, 2008.
GenBank No. ZP_01313517.1, ketol-acid reductoisomerase [Desulfuromonas acetoxidans DSM 684], May 15, 2006.
GenBank No. O82043, Ketol-acid reductoisomerase, chloroplastic, Jun. 16, 2009.
GenBank No. NP_977840.1, ketol-acid reductoisomerase [Bacillus cereus ATCC 10987], May 1, 2009.
GenBank No. NP_978252.1, ketol-acid reductoisomerase [Bacillus cereus ATCC 10987], May 1, 2009.
GenBank No. P05793, Daniels, et al., Jun. 16, 2009; pp. 1-9.
Entrez GenBank Accession No. UNIPROT: Q6F821, Barbe, et al., Oct. 2004; pp. 1-2.
GenBank Accession No. Q4K608, ketol-acid reductoisomerase, Pseudomonas fluorescens, Aug. 2, 2005, viewed Nov. 14, 2008.
GenBank: EDR97797.1, ketol-acid reductoisomerase [Anaerostipes caccae DSM 14662], Aug. 4. 2012.
Vasantha, et al., Genes for Alkaline Protease and Neutral Protease from Bacillus amyloliquefaciens Contain a Large Open Reading Frame Between the Regions Coding for Signal Sequence and Mature Protein, J. Bacteriol., 159:811-819, 1984.
Pearson, Comput. Methods Genome Res., [Proc. Int. Symp.] (1994), Meeting Date 1992, 111-20. Editor(s): Suhai, Sandor. Plenum: New York, NY.
Altschul, et al., Basic Local Alignment Search Tool, J. Mol. Biol. 215:403-410, 1990.
Spano, et al., Environmental stress response in wine lactic acid bacteria: beyond Bacillus subtilis, Crit. Rev. Microbiol. 32:77-86, 2006.
Chang, et al., Construction and Characterization of Amplifiable Multicopy DNA Cloning Vehicles Derived from the P15A Cryptic Miniplasmid, J. Bacteriol. 134:1141-1156, 1978.
Ferain, et al., Lactobacillus plantarum IdhL gene: overexpression and deletion, J. Bacteriol. 176:596-601, 1994.
Godon. et al., Branched-chain amino acid biosynthesis genes in *Lactococcus lactic* subsp. *lactis*, J. Bacteriol. 174:6580-6589, 1992.
Horinouchi, et al., Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics, J. Bacteriol. 150:804-814, 1982.
Higgins, et al., CLUSTAL V: improved software for multiple sequence alignment, CABIOS 8:189-191, 1992.
Higgins et al., Fast and sensitive multiple sequence alignments on a microcomputer, CABIOS Communications 5:151-153, 1989.
Johnson, et al., DNA sequences at the ends of transposon Tn5 required for transposition, Nature 304:280-282, 1983.
Rud, et al., A synthetic promoter library for constitutive gene expression in lactobacillus plantarum, Microbiol. 152:1011-1019, 2006.

(56) **References Cited**

OTHER PUBLICATIONS

Yansura, et al., Use of the *Escherichia coli* lac repressor and operator to control gene expression in Bacillus subtilis, Proc. Natl. Acad. Sci. USA, vol. 81:439-443, 1984.

Yuan, et al., Regulation of groE Expression in Bacillus subtills: the Involvement of the cr-Like Promoter and the Roles of the Inverted Repeat Sequence (CIRCE), J. Bacteriol. 177:5427-5433, 1995.

Methods in Yeast Genetics, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202.

Butanols, Ullman's Encyclopedia of Industrial Chemistry, 6th Edition, 5:716-719, 2003.

GenBank Accession No. B9CVH4, viewed Feb. 8, 2011.

GenBank Accession No. NC_000913, *Escherichia coli* str. K-12 substr. MG1655, May 17, 2008.

GenBank Accession No. NC_002505, Vibrio cholerae O1 biovar eltor str. N16961, Jul. 21, 2008.

GenBank Accession No. AAU36450, Psuedomonas aeruginosa cellular proliferation protein, Feb. 14, 2002, viewed Nov. 14, 2008.

GenBank Accession No. A3EGY6, ketol-acid reductoisomerase, Vibrio chlorea, Mar. 20, 2007, viewed Nov. 14, 2008.

GenBank Accession No. YP_162876, ketol-acid reductoisomerase [*Zymomonas mobilis* subsp. *mobilis ZM4* = ATCC 31821], Jun. 10, 2013.

GenBank Accession No. ZP_07930881, ketol-acid reductoisomerase [*Anaerostipes* sp. 3_2_56FAA], Nov. 27, 2012.

Carlquist, Magnus et al., Genetically engineered *Saccharomyces cerevisiae* for kinetic resolution of racemic bicyclo[3.3.1]nonane-2,6-dione, Tetrahedron: Asymmetry, 2008, pp. 2293-2295, vol. 19.

Eglinton, Jeffrey M. et al., Decreasing acetic acid accumulation by a glycerol overproducing strain of *Saccharomyces cerevisiae* by deleting the ALD6 aldehyde dehydrogenase gene, Yeast, 2002, pp. 295-301, vol. 19.

Fujisawa, Hisae et al., Characterization of short-chain dehydrogenase/reductase homologues of *Escherichia coli* (YdfG) and *Saccharomyces cerevisiae* (YMR226C), Biochimica et Biophysica Acta, 2003, pp. 89-94, vol. 1645.

Johanson, Ted et al., Strain engineering for stereoselective bioreduction of dicarbonyl compounds by yeast reductases, FEMS Yeast Research, 2005, pp. 513-525, vol. 5.

Overcamp, Karin M. et al., Metabolic Engineering of Glycerol Production in *Saccharomyces cerevisiae*, Applied and Environmental Microbiology, Jun. 2002, pp. 2814-2821, vol. 68, No. 6.

Wurdig, G. et al., Occurrence, detection, and determination of 2- and 3-methyl-2,3-dihydroxybutyric acid and 2-hydroxyglutaric acid in wine, From the Wine Research Institute of the State Viticultural, Horticultural, and Agricultural Training and Research Center, Trier, 1969, pp. 216-230, Vitis 8.

Yang, Yan et al., Enzymatic ketone reduction: mapping the substrate profile of a short-chain alcohol dehydrogenase (YMR226c) from *Saccharomyces cerevisiae*, Tetrahedron: Asymmetry, 2007, pp. 1799-1803, vol. 18.

Katz, Michael et al., Efficient Anaerobic Whole Cell Stereoselective Bioreduction With Recombinant *Saccharomyces cerevisiae*, Biotechnology and Bioengineering, Dec. 5, 2003, pp. 573-582, vol. 84, No. 5.

Bianci, Michele M. et al., The 'petite-negative' yeast Kluyveromyces lactis has a single gene expressing pyruvate decarboxylase activity, Molecular Microbiology, 1996, pp. 27-36, vol. 19, No. 1.

Hohmann, Stefan, Characterisation of PDC2, a gene necessary for high level expression of pyruvate decarboxylase structural genes in *Saccharomyces cerevisiae*, Mol. Gen. Genet., 1993, pp. 657-666, vol. 241.

Saint-Prix, Florence et al., Functional analysis of the ALD gene family of *Saccharomyces cerevisiae* during anaerobic growth on glucose: the NADP+-dependent Ald6p and Ald5p isoforms plays a major role in acetate formation, Microbiology, 2004, pp. 2209-2220, vol. 150.

Johnson, Monique A. et al., Positive Selection of Novel Peroxisome Biogenesis-Defective Mutants of the Yeast *Pichia pastoris*, Genetics, Apr. 1999, pp. 1379-1391, vol. 151.

Wang, Xinping et al., Molecular Cloning, Characterization, and Potential Roles of Cytosolic and Mitochondrial Aldehyde Dehydrogenases in Ethanol Metabolism in *Saccharomyces cerevisiae*, Journal of Bacteriology, Feb. 1998, pp. 822-830, vol. 180, No. 4.

Navarro-Avino, Juan P. et al., A Proposal for Nomenclature of Aldehyde Dehydrogenases in *Saccharomyces cerevisae* and Characterization of the Stress-Inducible ALD2 and ALD3 Genes, Yeast, 1999, pp. 829-842, vol. 15.

Nevoigt, Elke et al., Reduced Pyruvate Decarboxylase and Increased Glycerol-3-phosphate Dehydrogenase [NAD+] Levels Enhance Glycerol Production in *Saccharomyces cerevisiae*, Yeast, 1996, pp. 1331-1337, vol. 12.

Flikweert, Marcel T. et al., Pyruvate Decarboxylase: An Indispensable Enzyme for Growth of *Saccharomyces cerevisiae* on Glucose, Yeast, 1996, pp. 247-257, vol. 12.

Nomura, Yukihiro et al., Preparation and Preservation of Freeze-dried Cells of Acetic Acid Bacteria with Aldehyde Oxidase Activity, Bioscience Biotechnology Biochemistry, 1998, pp. 1134-1137, vol. 62, No. 6.

Kaneko, Tetsuo et al., Secoiridoid and Flavonoid Glycosides From Gonocaryum Calleryanum, Phytochemistry, 1995, pp. 115-120, vol. 39, No. 1.

Bakker, Barbara M. et al., Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*, FEMS Microbiology Reviews, 2001, pp. 15-37, vol. 25.

Kumanovics, et al. Identification of FRA 1 and FRA2 as genes involved in regulating the yeast iron regulon in response to decreased mitochondrial iron-sulfur cluster synthesis. J. Biol. Chem. 283:10276-10286, 2008.

* cited by examiner

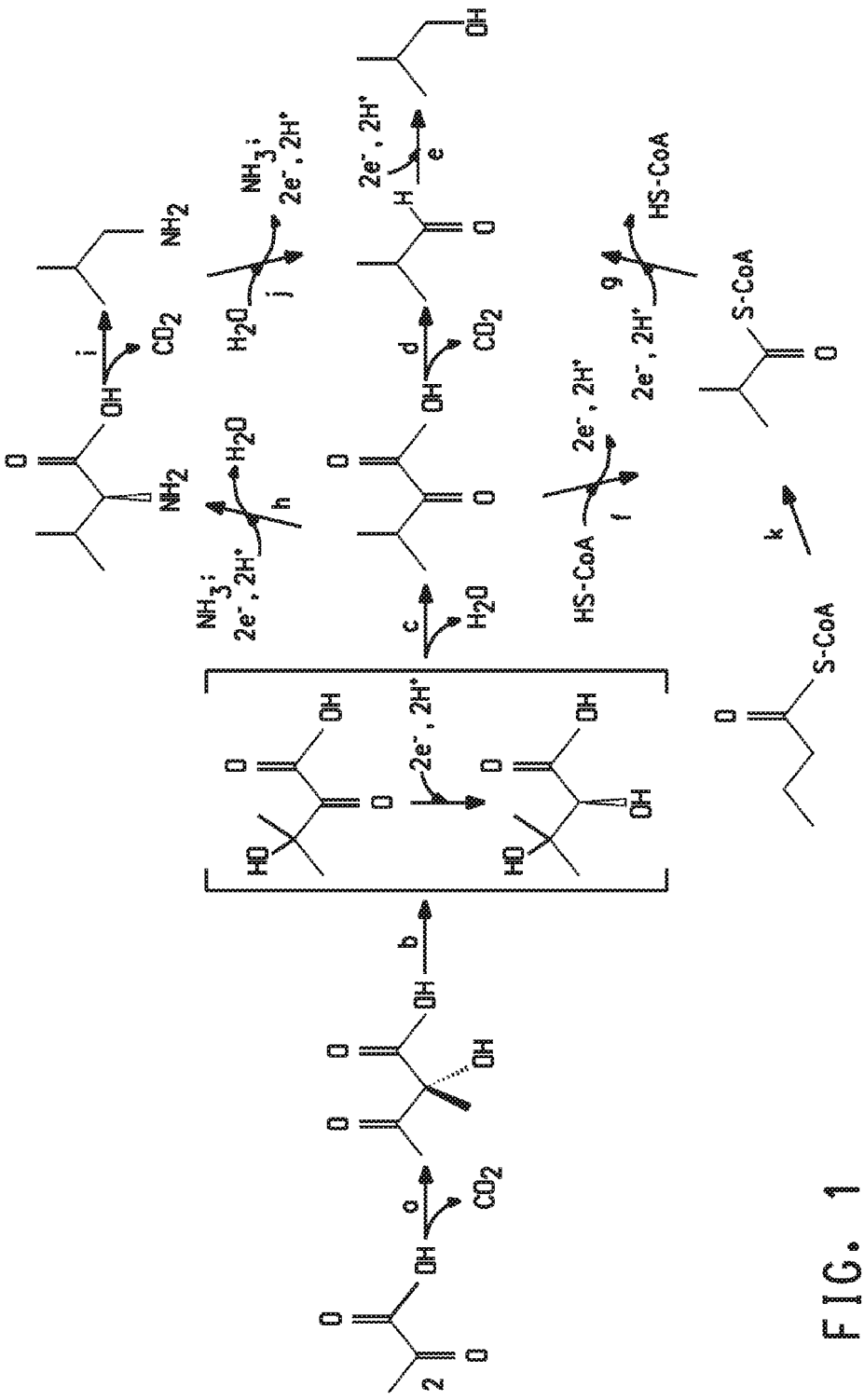


FIG. 1

```
K9     (1) MEECKMAKIYYQEDCNLSLLDGKTIAVIGYGSQGHAHALNAKESGCNVII
PF5    (1) -------MKVFYDKDCDLSIIQGKKVAIIGYGSQGHAQACNLKDSGVDVTV

K9    (51) GLYEGSKSWKRAEEQGFEVYTAAEAAKKADIIMILINDEKQATMYKNDIE
PF5   (45) GLRKGSATVAKAEAHGLKVTDVAAAVAGADLVMILTPDEFQSQLYKNEIE

K9   (101) PNLEAGNMLMFAHGFNIHFGCIVPPKDVDVTMIAPKGPGHTVRSEYEEGK
PF5   (95) PNIKKGATLAFSHGFAIHYNQVVPRADLDVIMIAPKAPGHTVRSEFVKGG

K9   (151) GVPCLVAVEQDATGKALDMALAYALAIGGARAGVLETTFRTETETDLFGE
PF5  (145) GIPDLIAIYQDASGNAKNVALSYAAGVGGGRTGIIETTFKDETETDLFGE

K9   (201) QAVLCGGVCALMQAGFETLVEAGYDPRNAYFECIHEMKLIVDLIYQSGFS
PF5  (195) QAVLCGGTVELVKAGFETLVEAGYAPEMAYFECLHELKLIVDLMYEGGIA

K9   (251) GMRYSISNTAEYGDYITGPKIITEDTKKAMKKILSDIQDGTFAKDFLVDM
PF5  (245) NMNYSISNNAEYGEYVTGPEVINAESRQAMRNALKRIQDGEYAKMFISEG

K9   (301) SDAGSQVHFKAMRKLASEHPAEVVGEEIRSLYSWSDEDKLINN---
PF5  (295) --ATGYPSMTAKRRNNAAHGIEIIGEQLRSMMPWIGANKIVDKAKN
```

FIG. 2

```
K9          (1) MEECKMAKIYYQEDCNLSLLDGKTIAVIGYGSQGHAHALNAKESG-CNVI
K1          (1) ------MAATIWYEKDADLSVFEGKKVAVIGYGSQGHAHALNLRDSG-VDVV
K2          (1) ------MAAQIWYENDCDLSVLDGKKVAIIGYGSQGHAHALNLRDSG-VDVV
K7          (1) ------MPKMYYEKDTDLNLLRGKKVAIIGYGSQGHAHALNLHESG-VDVV
S2          (1) -QEELMKVYYDSDADLGLIKSKKIAILGYGSQGHAHAQNLRDSGVAEVA
K25         (1) ------MVKVYYNEVTDN--LGDKTIAIIGYGSQGHAHAQNLRDTG-HNVV
K26         (1) ------MAVQMEYEKDVKVPALDGKKIAVIGYGSQGHAHSQNLRDTG-HDVI
L. Lactis   (1) ------MAVTMYYEDDVEVSALAGKQIAVIGYGSQGHAHAQNLRDSG-HNVI

K9         (50) IGLYEGSKSWKRAEEQGFEVYTAAEAAKKADIIMILIPDEKQATMYKNDI
K1         (46) VGLRPTSKSVEYAKEQGLEVQSVADATAEADVVMILLPDQYQAAVYKSEI
K2         (46) VGLRPTSKSVEQAKEQGLEVKSVPEAAAEADIIMILAPDQYQRTIWANDI
K7         (45) VGLYNGSKSWAKAFAAGLQVATVADAAKAADLIMILLPDEKQAKIYNEEI
S2         (50) IALRPDSASVKKAQDAGFKVLTNAEAAKWADILMILAPDEHQAAIYAEDL
K25        (43) IGIRAG-KSAENAKNDGFTVLPVSEAVQQANVVMILAPDEIQGELYEKEI
K26        (46) IGVRPG-KSFDKAKEDGFDTYTVAEATKLADVIMILAPDEIQQELYEAEI
L. Lactis  (46) IGVRHG-KSFDKAKEDGFETFEVGEAVAKADVIMVLAPDEIQQSIYEEDI

K9        (100) EPNLEAGNMLMFAHGFNIHFGCIVPPKDVDVTMIAPKGPGHTVRSEYEEG
K1         (96) EPNLKFGAALAFAHGFNIHYGYIKPSEDHPVFMVAPKGPGHIVRREYTNG
K2         (96) EPNIKFGAAIAFAHGFNIHYGYIKPSEDHPVFMVAPKGPGHIVRREYANG
K7         (95) APNLEEGNALVFAHGFNIHFGQVVPPSYVDVFMVAPKGPGHLVRRTYTEG
S2        (100) KDNLRFGSAIAFAHGLNIHFGLIEPRKDIDVFMIAPKGPGHTVRSEYVRG
K25        (92) APNLSAGDALAFAHGFNIHFEVITPPKEVDVFLVAPKGPGHLVRRTFSEG
K26        (95) APNLEAGNAVGFAHGFNIHFEFIKVPADVDVFMCAPKGPGHLVRRTFEEG
L. Lactis  (95) KPNLKAGSALGFAHGFNIHFGYIKVPEDVDVFMVAPKAPGHLVRRTYTEG

K9        (150) KGVPCLVAVEQDATGKALDMALAYALAIGGARAGVLETTFRTETETDLFG
K1        (146) RGVPVTVAVEQDPDGKTWPLCLAYAKALGALRAGAIKTTFEEETETDLFG
K2        (146) RGVPVVVAVEQDPRGDAWDTTLAYAKALGALRACAIKTTFKEETETDLFG
K7        (145) AGVPCLIAVHQDATGKAKQYALAYANGIGGARAGVLETTFKDETETDLFG
```

FIG. 3A

```
S2          (150)  GGVPCLVAVDQDASGNAHDIALAYASGIGGGRSGVIETTFREEVETDLFG
K25         (142)  FAVPALFAVYQDATGNAQETALSYAKGIGATRVGVLETTFKEETETDLFG
K26         (145)  FGVPALYAVYQDATGNAKDIAMDWCKGIGAAPVGLLETTYKEETEEDLFG
L. Lactis   (145)  FGTPALFVSHQNASGHAREIAMDWAKGIGCAKVGIIETTFKEETEEDLFG

K9          (200)  EQAVLCGGVCALMQAGFETIVEAGYDPRNAYFECIHEMKLIVDLIYQSGF
K1          (196)  EQDVLMGGINHLCDMGFDVLTEAGYQPEIAYFEVTHELKMLVDLANEGGL
K2          (196)  EQNVLMGGVNKLVEMGFEVLTDAGYQPEIAYFEVCHELKMLVDLMNEGGL
K7          (195)  EQAVLCGGVSELIKAGFETIVEAGYAPENAYFECMHEMKLIVDLIYQGGL
S2          (200)  EQAVLCGGLTALITAGFETLTEAGYAPEMAFFECMHEMKLIVDLIYEAGI
K25         (192)  EQAVLCGGLTSMIETGFETIVEAGYQPELAYFEVCHEMKLIVDLIYEGGF
K26         (195)  EQAVLCGGLTALIETGFEVLTEAGYAPELAYFEVLHEMKLIVDLIYEGGF
L. Lactis   (195)  EQAVLCGGLTALVEAGFETLTEAGYAGELAYFEVLHEMKLIVDLMYEGGF

K9          (250)  SGMRYSISNTAEYGDYITGPKLITEDTRKAMKKILSDIQDGTFAKDFIVD
K1          (246)  NKARWSCSDTAQYGDYTS--TVITEETKKRMQYQLKRIQDGSFAKEFMDD
K2          (246)  NKARWSCSDTAQYGDYTN--TVINEDCRKRMQYHLGRIQDGSFAKEFIDD
K7          (245)  SMMRYSISDTAEYGDYQIGRRITTDETKKEMKKVLTEIQDGTFAKNWLIE
S2          (250)  ANMRYSISNTAEYGDIVSGPRVINEESKRAMKAILDDIQSGRFVSKFVLD
K25         (242)  AKMRDSISNTAEYGDYVSGSRIITEQTKANMKNVLKDIQNGTFAKGFIAD
K26         (245)  KKMRQSISNTAEFGDYVSGPRVITEQVKENMKAVLADIQNGKFANDFVND
L. Lactis   (245)  TKMRQSISNTAEFGDYVTGPRIITDEVKKNMKLVLADIQSGKFAQDFVDD

K9          (300)  MSDAGSQVHFKAMRKLASEHPAEVVGEEIRSLYSWSDEDKLINN------
K1          (294)  QAAG--APKFKKLQEEYSHPHLETVGPKLRAMFSWNNQVDADADMAESFN
K2          (294)  QDAG--APKFKELQEEYGNVKLETVGPKLRAMFSWNNGQDDDADMAT-FT
K7          (295)  NQTN--RPGFNARRRMEAEHPIEKVGKELRGMMSWIDTAKVD--------
S2          (300)  NRAG--QPELKAARKRMAAHPIEQVGARLRKMMPWIASNKIVDKARN-KL
K25         (292)  NQAG--FPEFKKMREQNGNHPIEKVGSELRKMMPTVTKD----------
K26         (295)  YKAG--RPKLTAYREEAANLELEKVGAELRKAMPFVGQNDDDAFKIYN--
L. Lactis   (295)  FKAG--RPKLIAYREAAKNLEIEKIGAELRQAMPFTQSGDDDAFKIYQ--
```

FIG. 3B

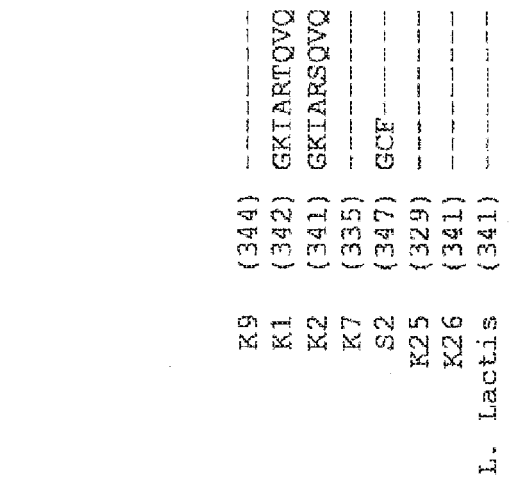

K9 (344) ----------
K1 (342) GKIARTQVQ
K2 (341) GKIARSQVQ
K7 (335) ----------
S2 (347) GCF-------
K25 (329) ----------
K26 (341) ----------
L. Lactis (341) ----------

FIG. 3C

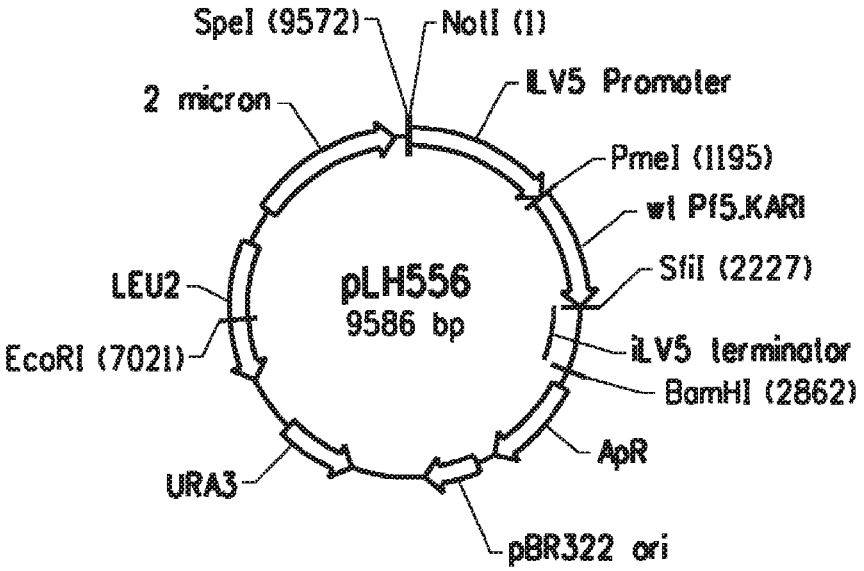

SpeI (9572)
NotI (1)
ILV5 Promoter
2 micron
PmeI (1195)
wt P15.KARI
SfiI (2227)
LEU2
pLH556
9586 bp
EcoRI (7021)
iLV5 terminator
BamHI (2862)
ApR
URA3
pBR322 ori

FIG. 4

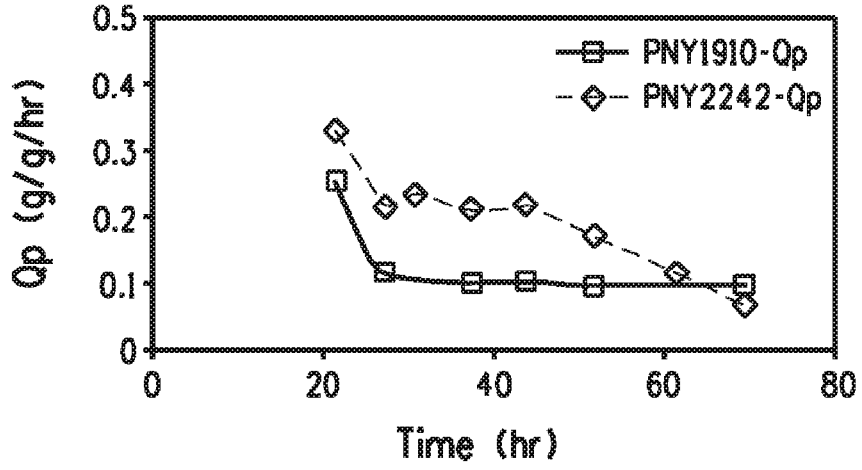

0.5
0.4
0.3
0.2
0.1
0
Qp (g/g/hr)
PNY1910-Qp
PNY2242-Qp
0
20
40
60
80
Time (hr)

FIG. 5

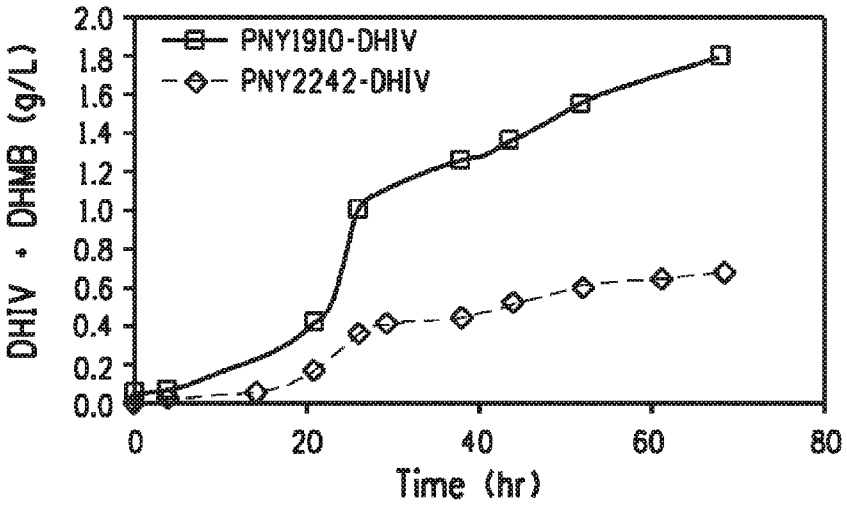

PNY1910-DHIV
PNY2242-DHIV
DHIV + DHMB (g/L)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
Time (hr)

FIG. 6

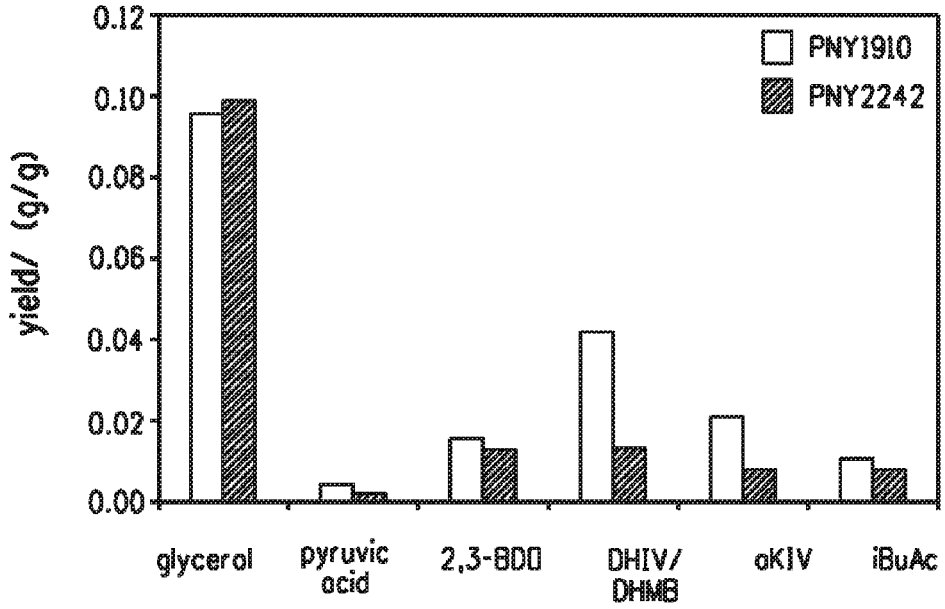

PNY1910
PNY2242
yield/ (g/g)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
glycerol
pyruvic acid
2,3-BDO
DHIV/ DHMB
αKIV
iBuAc

FIG. 7

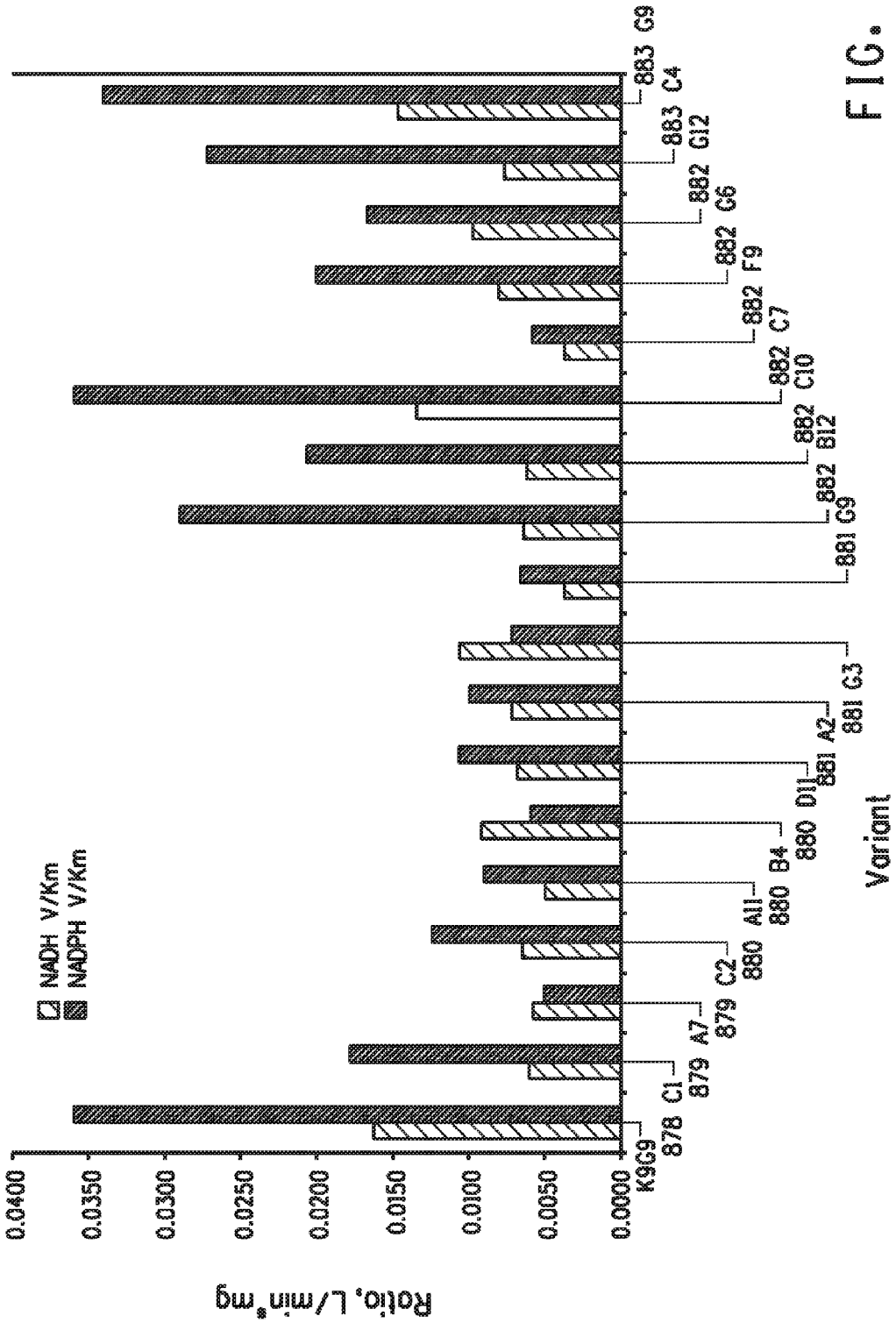

NADH V/Km
NADPH V/Km
Ratio, L/min*mg
0.0400
0.0350
0.0300
0.0250
0.0200
0.0150
0.0100
0.0050
0.0000
K9G9
878
C1
879
A7
879
C2
880
A11
880
B4
880
D11
881
A2
881
G3
881
C9
881
B12
882
C10
882
C7
882
F9
882
G6
882
G12
883
C4
883
G9
Variant

FIG. 8

FIG. 9A

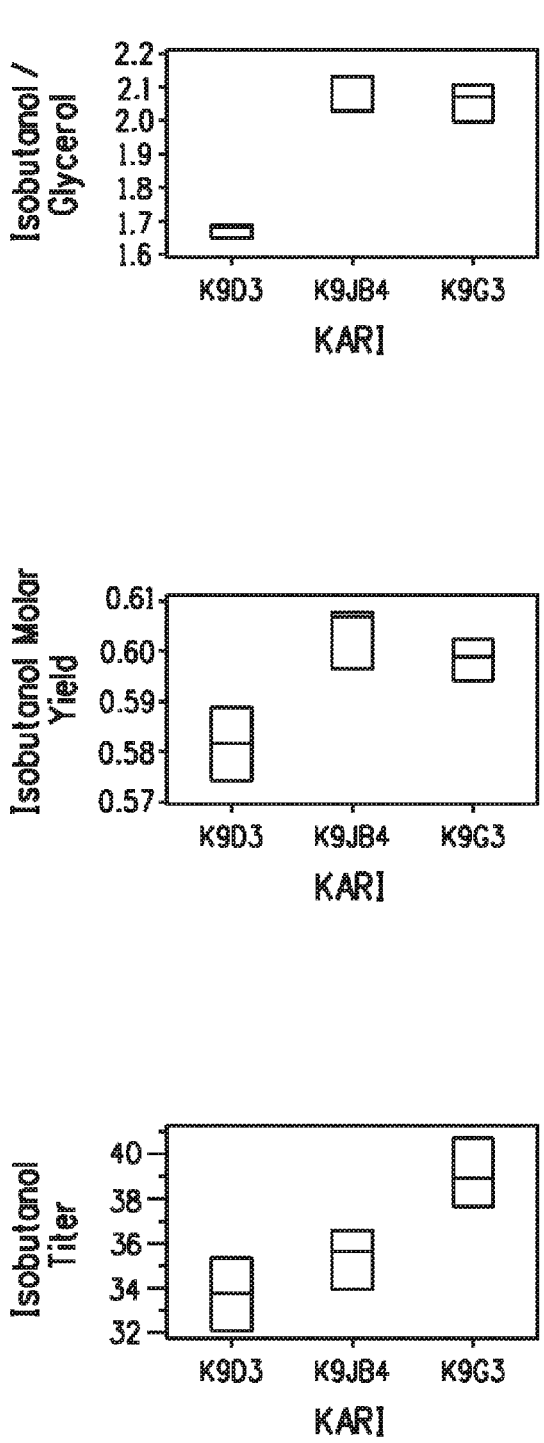

Isobutanol / Glycerol
2.2
2.1
2.0
1.9
1.8
1.7
1.6
K9D3
K9JB4
K9G3
KARI
Isobutanol Molar Yield
0.61
0.60
0.59
0.58
0.57
K9D3
K9JB4
K9G3
KARI
Isobutanol Titer
40
38
36
34
32
K9D3
K9JB4
K9G3
KARI

FIG. 9B

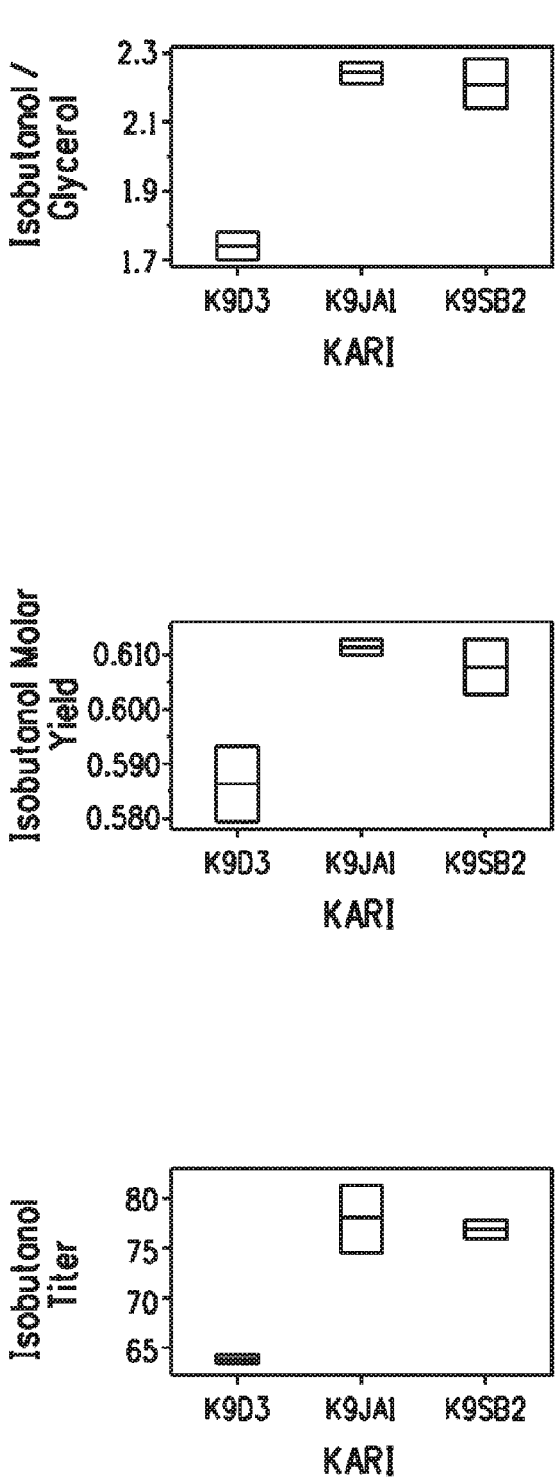
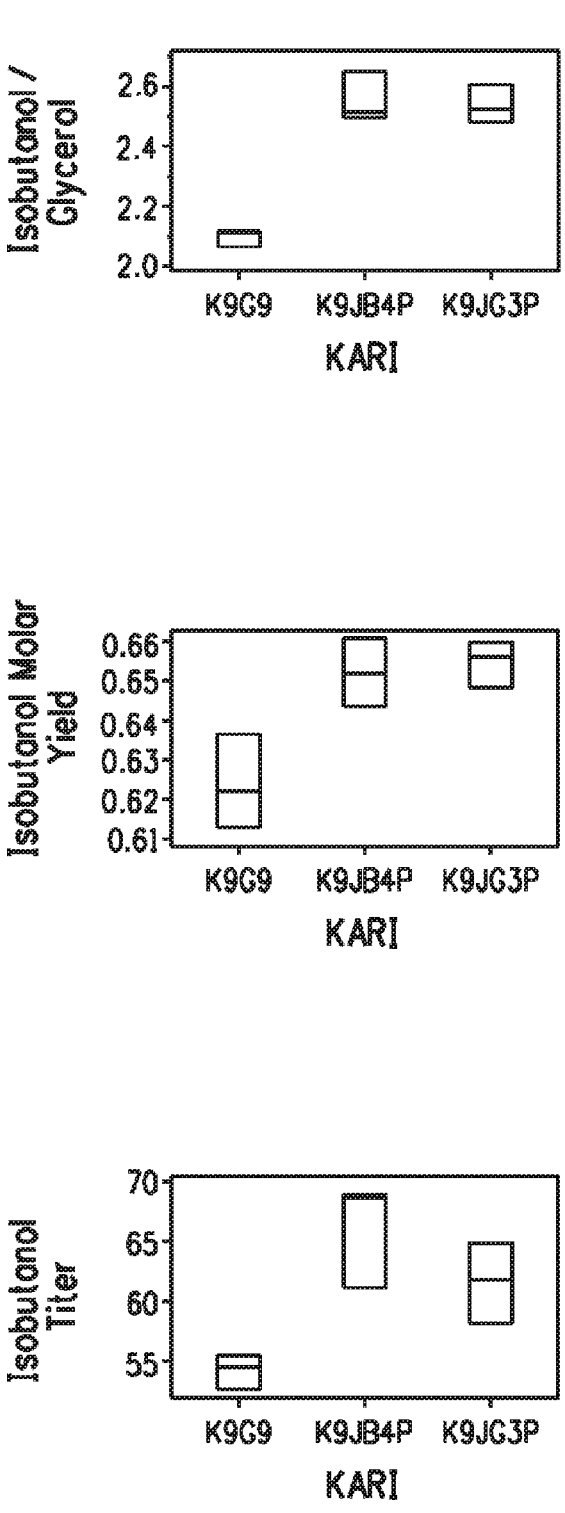

Isobutanol / Glycerol
2.3
2.1
1.9
1.7
K9D3
K9JAI
K9SB2
KARI
Isobutanol Molar Yield
0.610
0.600
0.590
0.580
K9D3
K9JAI
K9SB2
KARI
Isobutanol Titer
80
75
70
65
K9D3
K9JAI
K9SB2
KARI

FIG. 9C


Isobutanol / Glycerol
2.6
2.4
2.2
2.0
K9G9
K9JB4P
K9JG3P
KARI
Isobutanol Molar Yield
0.66
0.65
0.64
0.63
0.62
0.61
K9G9
K9JB4P
K9JG3P
KARI
Isobutanol Titer
70
65
60
55
K9G9
K9JB4P
K9JG3P
KARI

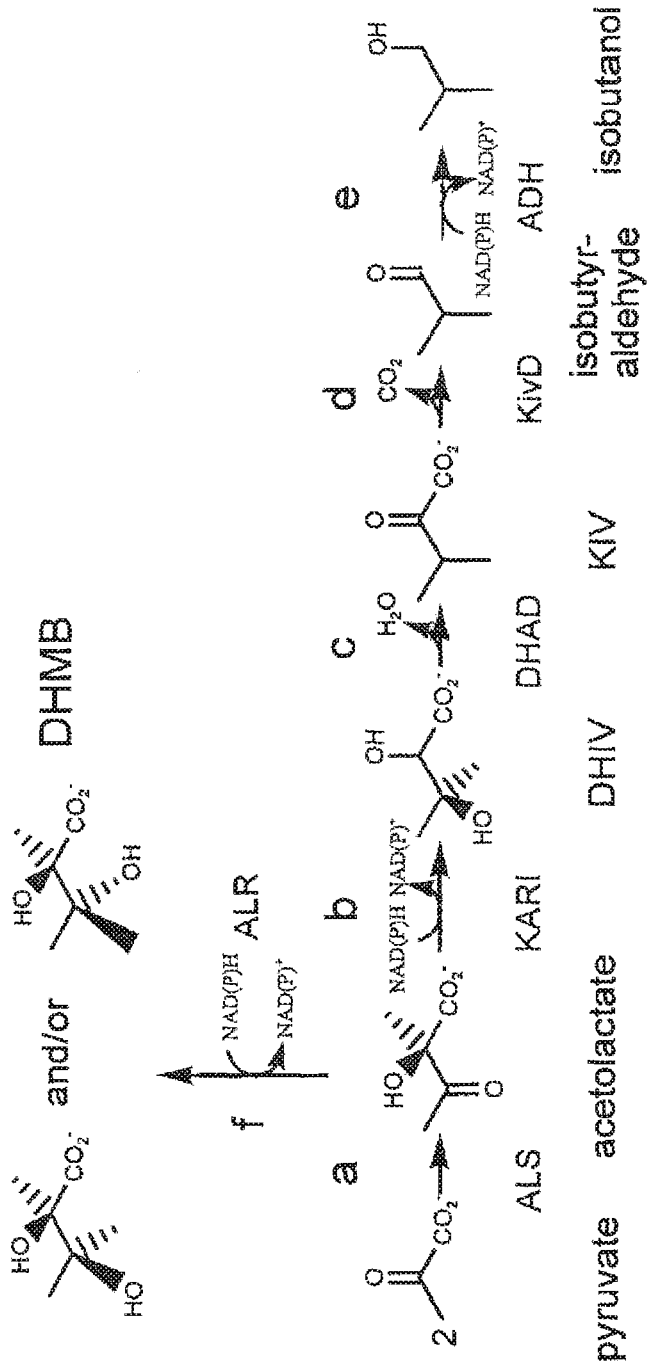

pyruvate
acetolactate
DHMB
and/or
ALS
KARI
DHAD
KIV
KivD
ADH
isobutyr-aldehyde
isobutanol
ALR
DHIV
a
b
c
d
e
f
2
NAD(P)H
NAD(P)+

FIG. 10

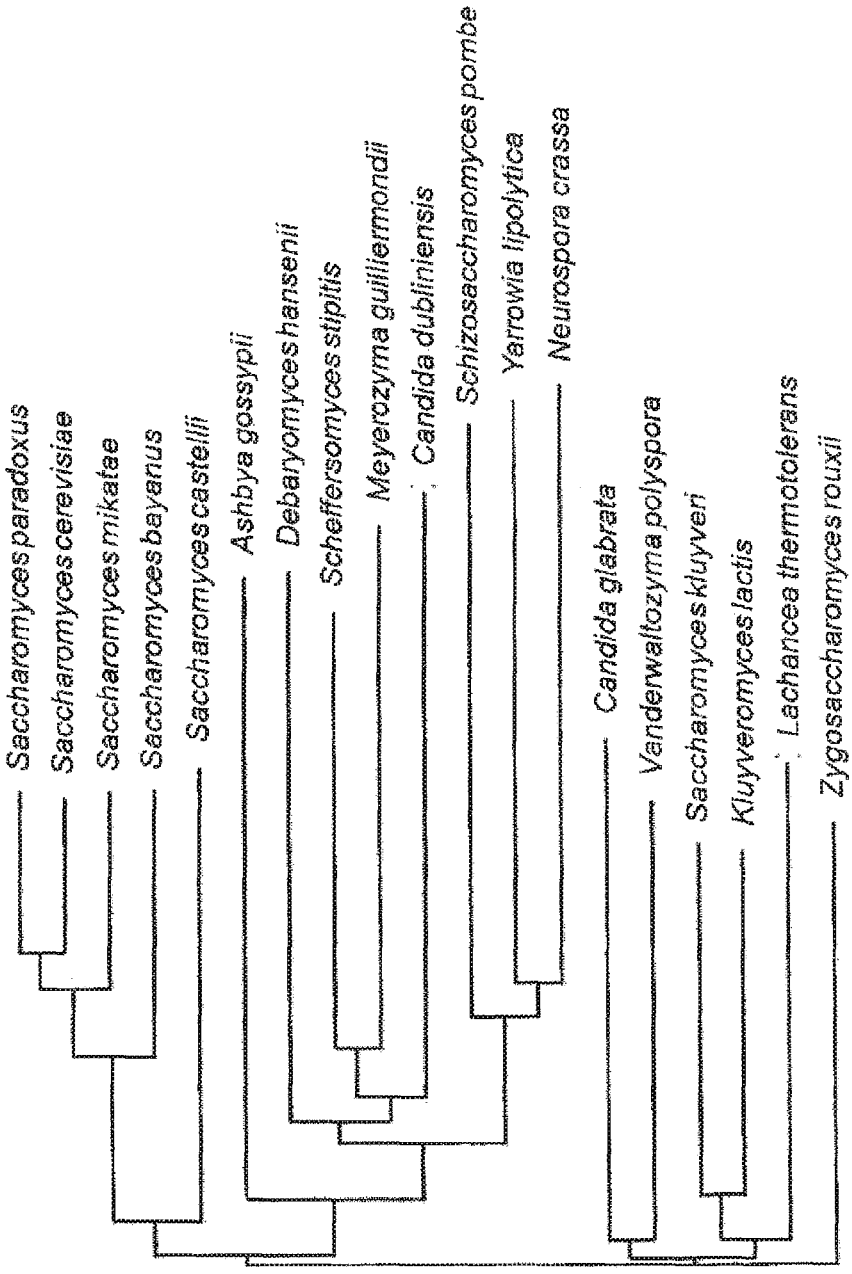

Saccharomyces paradoxus
Saccharomyces cerevisiae
Saccharomyces mikatae
Saccharomyces bayanus
Saccharomyces castellii
Ashbya gossypii
Debaryomyces hansenii
Scheffersomyces stipitis
Meyerozyma guilliermondii
Candida dubliniensis
Schizosaccharomyces pombe
Yarrowia lipolytica
Neurospora crassa
Candida glabrata
Vanderwaltozyma polyspora
Saccharomyces kluyveri
Kluyveromyces lactis
Lachancea thermotolerans
Zygosaccharomyces rouxii

FIG. 11

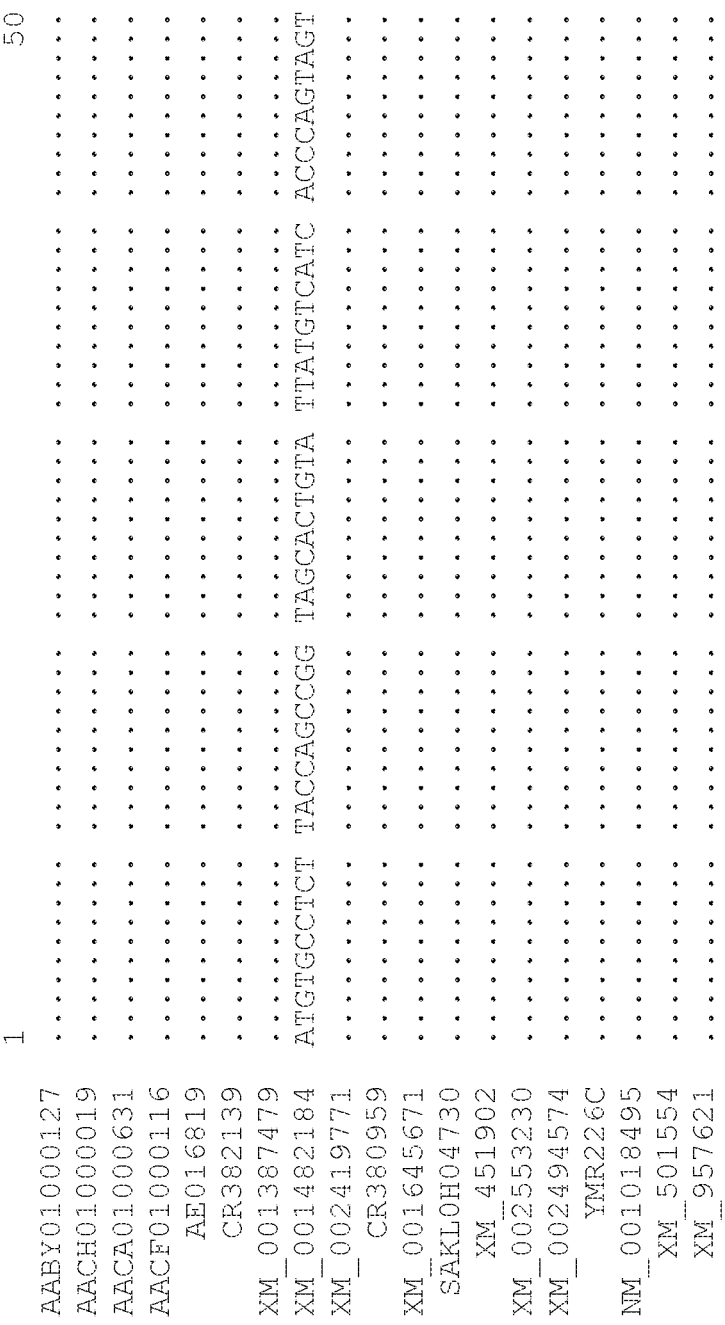

1
50
AABY01000127 ....
AACH01000019 ....
AACA01000631 ....
AACF01000116 ....
AE016819 ....
CR382139 ....
XM_001387479 ....
XM_001482184 ATGTGCCTCT TACCAGCCGG TAGCACTGTA TTATGTCATC ACCCAGTAGT
XM_002419771 ....
CR380959 ....
XM_001645671 ....
SAKL0H04730 ....
XM_451902 ....
XM_002553230 ....
XM_002494574 ....
YMR226C ....
NM_001018495 ....
XM_501554 ....
XM_957621 ....

FIG. 12A

```
              51                                             100
AABY01000127  .......... .......... ........AT GTCCCAAGGT AGAAAAGCTG
AACH01000019  .......... .......... ........AT GTCTCAAGGT AGAAAAGCTG
AACA01000631  .......... .......... ........AT GTCCCAAGGT AGAAAAGCTG
AACF01000116  .......... .......... ........AT GTCTCAAGGT CCTAAAGCTG
    AE016819  .......... .......... ........AT GTCCCTAGGA AGAAAAGCAG
    CR382139  .......... .......... ........AT GTCGTACGGA TCTAAAGCTG
XM_001387479  .......... .......... ........AT GTCGTTTGGA AAAAAAGCTG
XM_001482184  GAGTGTGGAG ATTAAATCCT CAATCTTCAT GTCTTTCGGT GCCAAAGCCG
XM_002419771  .......... .......... ........AT GTCATTTGGT AGAAAAGCTG
    CR380959  .......... .......... ........AT GTCTCAAGGA AGAAAAGCTG
XM_001645671  .......... .......... ........AT GTCACAGGGT AGAAAGGCTT
 SAKL0H04730  .......... .......... ........AT GTCTCAAGGT AGAAGGGCTG
   XM_451902  .......... .......... ........AT GTCTCAAGGT AGAAAGGCTG
XM_002553230  .......... .......... ........AT GTCACAGGGA AGAAGAGCAG
XM_002494574  .......... .......... ........AT GTCACAAGGT GTCAAAGCTG
     YMR226C  .......... .......... ........AT GTCCCAAGGT AGAAAAGCTG
NM_001018495  .......... .......... .......... .......... ..........
   XM_501554  .......... .......... ........AT GTCTTTCGGA GATAAAGCTG
   XM_957621  .......... .......... ........AT GTCCTCAGC. .....AGTAG
```

FIG. 12B

```
               101                                              150
AABY01000127   CAGAAA.GAT TGGCTAACAA GACCGTGCTC ATTACGGGTG CCTCTGCTGG
AACH01000019   CAGAAA.GAT TGGCTGGCAA AACCGTTCTC ATCACGGGTG CCTCTGCTGG
AACA01000631   CAGAAA.GAT TGGCCAACAA GACGGTGCTC ATTACAGGCG CTTCTGCTGG
AACF01000116   CCGAAA.GAT TGAATGAGAA GATTGTGTTT ATCACTGGTG CTTCAGCTGG
    AE016819   CTGAAA.GAT TAGCCAACAA AATTGTGCTT GTGACTGGTG CCTCTGCGGG
    CR382139   CTGAAC.GTG TTGCCAATAA GATTGTCTTA ATCACTGGTG CTTCATCTGG
XM_001387479   CTGAAA.GAC TTGCCAACAA AATCATTCTT ATCACCGGGG CTTCGTCTGG
XM_001482184   CTGAAC.GCC TTGCCAACAA GATCATATTG ATCACTGGGG CATCGTCTGG
XM_002419771   CTGAAA.GAT TAGCCAATAG ATCCATTCTT ATCACTGGTG CTTCATCTGG
    CR380959   CTGAGA.GGT TACAAGGGAA GATTGCCTTT ATTACGGGTG CCTCTGCGGG
XM_001645671   CAGAAA.GGT TGGCTGGTAA AACTGTATTA ATTACAGGTG CTTCATCAGG
 SAKL0H04730   CAGAAA.GAC TAGCAAACAA GACCGTTTTT ATAACTGGCG CCTCTGCCGG
   XM_451902   CTGAAA.GAT TGCAAAACAA GACAATTTTC ATTACCGGTG CTTCTGCAGG
XM_002553230   CTGAAA.GAC TGGCAGGAAA GACTGTCTTC ATCACAGGCG CATCAGCCGG
XM_002494574   CTGAAA.GAC TAGCTGGTAA GACTGTATTC ATTACAGGTG CTTCTGCAGG
     YMR226C   CAGAAA.GAT TGGCTAAGAA GACTGTCCTC ATTACAGGTG CATCTGCTGG
NM_001018495   ATGAGCCGTT TGGATGGAAA AACGATTTTA ATCACTGGTG CCTCTTCTGG
   XM_501554   CTGCTC.GAC TTGCGGGCAA GACCGTCTTT GTTACCGGCG CCTCGTCCGG
   XM_957621   CCAAGC.GCC TCGCGGGCAA GACCATTGTC ATAACCGGAG CGTCCTCAGG
```

FIG. 12C

	151				200
AABY01000127	TATTGGTAAG	GCCACCGCAT	TAGAGTATTT	G...GAGGCA	TCCAATGGTG
AACH01000019	TATTGGCAAA	GCCACTGCAT	TAGAGTATTT	G...GAGGCA	TCCAATGGCG
AACA01000631	TATTGGTAAG	GCCACCGCAT	TGGAGTATTT	G...GAAGCA	TCCAATGGAA
AACF01000116	TATTGGGCAA	GCCACCGCTT	TGGAATACAT	G...GATGCG	TCGAACGGTA
AE016819	CATTGGCCGT	GCTACAGCCA	TTAACTATGC	A...GACGCG	ACGGACGGGG
CR382139	AATTGGTGAA	GCAACTGCCA	AAGAAATTGC	A...TCAGCC	GCTAATGGCA
XM_001387479	TATTGGTGAA	GCTACAGCTA	GAGAGTTTGC	A...TCTGCT	GCCAATGGGA
XM_001482184	TATAGGCGAG	GCTACCGCCA	GAGAATTCGC	T...GCTGCT	GCCAATGGAA
XM_002419771	GATTGGTGAA	GCATGTGCTA	AAGTTTTCGC	T...GAAGCA	TCTAATGGTC
CR380959	CATCGGTAAA	GCTACAGCCA	TTGAGTATTT	G...GATGCT	TCCAATGGTA
XM_001645671	GATTGGGAAA	GCCACTGCAT	TAGAATATCT	A...GATGCC	TCCAATGGTC
SAKL0H04730	CATTGGCCAA	GCTACTGCTT	TGGAATACTG	T...GATGCT	TCTAACGGTA
XM_451902	TATTGGTCAA	GCCACAGCAT	TGGAATATCT	A...GATGCT	GCTAACGGTA
XM_002553230	TATCGGTCAG	GCCACTGCGC	AAGAATACCT	G...GAAGCA	TCCGAAGGCA
XM_002494574	TATCGGTCAA	GCAACTGCAA	AGGAATATTT	G...GATGCA	TCCAATGGTC
YMR226C	TATTGGTAAG	GCGACCGCAT	TAGAGTACTT	G...GAGGCA	TCCAATGGTG
NM_001018495	AATTGGAAAA	AGCACTGCTT	TTGAAATTGC	C...AAAGTT	GCCAAAG...
XM_501554	CATTGGCCAG	GCCACTGTTC	TCGCTCTAGC	C...GAAGCT	GCCAAGGGCG
XM_957621	AATCGGGCGT	AGCACAGCCT	TCGAGTTTGC	GCGGACCGCG	CCCAACCACG

FIG. 12D

```
                201                                                 250
AABY01000127 ATATGAAACT GGTCTTAGCT GCTAGAAGAT TAGAAAAGCT CGAGGAATTA
AACH01000019 ATATGAAATT AATCTTAGCC GCTAGAAGAT TAGAAAAGCT CGAGGAATTG
AACA01000631 ACATGAAACT GATCTTGGCT GCGAGGAGAT TGGAGAAGCT AGAGGAGCTG
AACF01000116 CTGTGAAATT GGTTCTAGTT GCCAGAAGAT TGGAGAAATT ACAACAATTG
        AE016819 CAATCAAGTT GATTTTGGTG GCAAGACGCG CAGAAAAGCT CACCAGCTTG
          CR382139 ATTTAAAATT AGTGTTGTGT GCTAGACGAA AAGAAAAGTT GGATAATTTA
XM_001387479 ATATCAGATT GATTTTGACA GCCAGAAGAA AAGAAAAGTT GGCTCAATTG
XM_001482184 AAATTCTGTT GATTTTGACC GCTCGGAGAG AAGACAAACT CAAGTCTCTC
XM_002419771 AAGTTAAATT AGTTTTAGGA GCAAGAAGAA AAGAACGATT AGTTAAATTA
          CR380959 GTGTGAAGCT AGTTCTTGGT GCACGTAGAA TGGAGAAATT GGAGGAGTTG
XM_001645671 ATATGAAGTT AATTTTAGTT GCAAGAAGAT TAGAAAAATT GCAAGAGTTG
  SAKL0H04730 AAATAAACTT GGTGTTAAGT GCCAGAAGGC TGGAAAAATT GCAAGAGTTA
     XM_451902 ATGTCAAATT GATCTTAGCA GCAAGAAGGT TGGCTAAGTT GGAAGAATTG
XM_002553230 AAATCAAGTT GATCCTTGCA GCAAGAAGAC TCGACAAGCT GGAGGAAATC
XM_002494574 AAATTAAATT GATCTTGGCT GCAAGAAGAT TAGAGAAATT ACACGAGTTT
          YMR226C ATATGAAACT GATCTTGGCT GCTAGAAGAT TAGAAAAGCT CGAGGAATTG
NM_001018495 ...TAAAACT TATTTTGGCT GCTCGCAGAT TTTCTACCGT TGAAGAAATT
     XM_501554 ACCTCAAGTT TGTGCTTGCT GCCCGACGAA CCGACCGTCT GGACGAGCTC
     XM_957621 GCCTCAAGCT GATCTTGACG GCCCGCCGCG TCGATGCCCT GGAGCAGATT
```

FIG. 12E

```
                  251                                                 300
AABY01000127 AAGAAAACTA TTGATCAGG. AGTTTCCA.. AACGCCAAAG TTCATGTGGC
AACH01000019 AAGAAGACTA TCGATGAAG. AGTTTCCA.. AACGCAAAGG TCCATGTGAC
AACA01000631 AAGAAGACCA TCGACGAGG. AGTTTCCC.. AATGCAAAGG TTCACGTTGG
AACF01000116 AAGGAAGTCA TTGAGGCAA. AATACCCT.. AAGAGTAAAG TCTATATTGG
      AE016819 AAACAGGAGA TCGA.AAGCA AGTATCCC.. AACGCCAAGA TCCATGTCGG
      CR382139 TCT.AAAGAA TTGACTGACA AATATTCA.. TCCATCAAGG TTCATGTTGC
XM_001387479 TCA.GACTCA TTGACCAAGG AATTTCCA.. ACTATCAAAA TCCATTCTGC
XM_001482184 TCG.CAACAA TTGAGCCTCA TTTACCCG.. CAAATTAAAA TCCATTCTGC
XM_002419771 TCT.GATACT TTAATTAAAC AATATCCT.. AATATTAAAA TTCATCATGA
      CR380959 AAG.AAGGAA TTGCTGGCTC AATATCCT.. GATGCAAAGA TTCATATAGG
XM_001645671 AAGGAAACAA TTTGTAAAG. AATATCCA.. GAATCTAAGG TTCATGTTGA
   SAKL0H04730 AAGGACAAAA TCACCAAGG. AGTATCCT.. GAAGCCAAGG TTTATATTGG
      XM_451902 AAGGAAAAAA TCAATGCTG. AATACCCA.. CAAGCTAAAG TATATATCGG
XM_002553230 AAAGCCAAGG TTTCTAAAG. ACTTCCCT.. GAAGCACAGG TGCATATCGG
XM_002494574 AAAGAACAAA CTAC.AAAGA GTTACCCA.. AGCGCTCAAG TCCACATTGG
      YMR226C AAGAAGACCA TTGATCAAG. AGTTTCCA.. AACGCAAAAG TTCATGTGGC
NM_001018495 GCAAAGGAGT TAGA.ATCGA AATAT..... GAAGTATCGG TTCTTCCTCT
      XM_501554 AAGAAGAAGC TGGAGACCG. ACTACAAG.. GGTATCCAGG TGCTGCCTTT
      XM_957621 GCCAAGGAGA TCCGCCAAGA GGTTGGCGAG GGCGTGCAAG TGCTGCCTGT
```

FIG. 12F

```
                301                                                 350
AABY01000127 CCAACTGGAT ATCACTCAAG CAGAAAAGAT CAAGCCCTTT ATTGAGAATT
AACH01000019 CAAACTGGAC ATCACACAGA CAGAAAAGAT CAAGCCCTTT ATTGAAAACT
AACA01000631 CCAACTGGAT ATCACACAGG CCGAGAAGAT CAAGCCCTTC ATTGAAAACT
AACF01000116 GAAGTTGGAT GTGACAGAGC TTGAGACCAT TCAACCATTC TTGGATAATC
      AE016819 ACAATTGGAT GTGACCCAAC TGGACCAGAT CCGCCCATTT TTGGAGGGAC
        CR382139 TCAACTAGAT GTATCTAAGC TCGAGACTAT CAAGCCATTT ATCAATGATT
XM_001387479 CAAATTGGAT GTGACCGAAC ATGATGGCAT CAAGCCTTTC ATTTCTGGTT
XM_001482184 TCGTCTTGAT GTCTCTGAGT TTTCGTCACT TAAGCCGTTC ATTACTGGGT
XM_002419771 TTTTTTGGAT GTTACTATTA AAGATTCAAT TTCAAAATTC ATTGCTGGAA
        CR380959 TAAACTGGAT GTTACAGACT TTGAAAACGT CAAGCAGTTT TTGGCTGACT
XM_001645671 AGAATTAGAT ATTTCTGATA TTAATAGAAT CCCAGAATTT ATTGCAAAAT
  SAKL0H04730 TGTGCTTGAT GTGACCGAAA CGGAAAAAAT CAAACCATTC TTGGATGGTT
      XM_451902 TCAATTGGAC GTCACTGAAA CTGAGAAGAT TCAACCTTTC ATTGATAACT
XM_002553230 CCAGCTAGAT GTGACTCAGA CGGACAAAAT CCAGCCTTTT GTCGACAATT
XM_002494574 TAAATTGGAC GTCACTGCAA TTGACACCAT AAAACCATTT TTGGATAAAT
        YMR226C CCAGCTGGAT ATCACTCAAG CAGAAAAAAT CAAGCCCTTC ATTGAAAACT
NM_001018495 TAAATTGGAT GTTTCTGATT TGAAGTCTAT TCCTGGGGTA ATTGAGTCAT
      XM_501554 CAAGCTGGAC GTGTCCAAGG TCGAGGAGAC CGAGAACATT GTGTCCAAGC
      XM_957621 CAAGCTGGAC GTCAGCCAGC CGGAGGAGGT CAGGGGGTTT GTTGGGAACT
```

FIG. 12G

```
                351                                                 400
AABY01000127 TGCCAAAGGA GTTCAAAGAC ATTGACATTT TGGTGAACAA CGCTGGGAAG
AACH01000019 TGCCAGAGGA GTTCAAAGAC ATTGATATTC TGGTGAACAA CGCTGGTAAG
AACA01000631 TGCCGGAGGC ATTCAAGGAT ATTGACATCC TGATAAACAA TGCCGGCAAA
AACF01000116 TTCCTGAGGA ATTTAAGGAT ATTGATATCT TGATTAATAA TGCCGGGAAG
     AE016819 TACCTGAGGA GTTCCGAGAC ATTGATATTT TAATTAACAA CGCAGGTAAG
       CR382139 TACCGAAAGA ATTCTCTGAC GTGGATGTAT TAGTCAACAA TGCAGGCTTG
XM_001387479 TACCCAAGGA TTTCGCCGAC ATCGATGTGT TGATCAACAA TGCTGGAAAA
XM_001482184 TGCCAAAGGA TTTTGCTAGC ATCGACGTTT TGGTGAATAA TGCGGGGAAA
XM_002419771 TTCCTCATGA ATTTG..AAC CT.GATGTAT TAATTAATAA TAGTGGTAAA
       CR380959 TGCCAGAAGA GTTCAAGGAC ATCGACATCC TGATCAATAA CGCTGGTAAA
XM_001645671 TACCTGAAGA ATTCAAAGAT ATTGATATAT TGATTAACAA TGCAGGTAAA
  SAKL0H04730 TACCAGAAGA ATTTAAAGAT ATTGACATCT TGATCAATAA TGCAGGCAAA
     XM_451902 TGCCGGAAGA ATTCAAGGAT ATCGATATTT TGATTAACAA TGCCGGTAAA
XM_002553230 TGCCCGAAGA GTTCAAAGAC ATCGACATCC TGATCAACAA CGCGGGCAAG
XM_002494574 TACCAAAGGA ATTTCAAGAT ATCGATATTT TGATCAACAA TGCCGGTAAG
       YMR226C TGCCACAAGA GTTCAAGGAT ATTGACATTC TGGTGAACAA TGCCGGAAAG
NM_001018495 TGCCAAAGGA ATTTGCTGAT ATCGATGTCT TGATTAATAA TGCTGGACTT
     XM_501554 TGCCCAAGGA GTTTTCCGAG GTGGACGTGC TTATCAACAA CGCCGGCATG
     XM_957621 TGCCCGAGGA GTGGAGGGAT ATCCATGTGC TGGTCAATAA TGCTGGTCTC
```

FIG. 12H

```
                401                                                 450
AABY01000127    GCCCTTGGTA CCGACCGTGT GGGGGAGATT GCAACACAAG ATATCCAGGA
AACH01000019    GCTCTTGGTA CGGACCGTGT TGGGGAGATT GATACACAGG ACGTCCAGGA
AACA01000631    GCCCTGGGCT CCGAACGTGT CGGGGAAATT GCCACACAGG ACATCCAGGA
AACF01000116    GCATTAGGTT CCGATCGTGT AGGTGATATT GATATAAAAG ATGTGAAGGG
     AE016819   GCCCTCGGCA CTGAGAGGGT GGGGGAAATC TCGATGGACG ATATCCAGGA
       CR382139 GCTTTGGGCC GTGATGAAGT TGGAACCATT GACACAGATG ATATGTTATC
XM_001387479    GCTCTTGGAA AAGCATCTGT TGGTGAAATC AGTGACAGTG ATATCCAAGG
XM_001482184    GCATTGGGAA GAGCCAATGT TGGTGAAATT TCCCAAGAGG AAATCAATGG
XM_002419771    GCCTTGGGGA AAGAAGAAGT TGGAGAATTG AAAGATGAAG ATATTACGGA
       CR380959 GCGTTGGGGT CTGACAAAGT TGGAGACATT GACCCTGAGG ATATCGCAGG
XM_001645671    GCATTAGGAA GTGATACTAT TGGTAATATC GAGAATGAGG ATATTAAAGG
  SAKL0H04730   GCGTTAGGCT CTGATCCTGT TGGTACCATC AAAACTGAAG ATATTGAAGG
     XM_451902  GCTTTGGGAT CTGATGTTGT CGGTACCATC AGTAGCGAGG ACATCAAAGG
XM_002553230    GCGCTCGGAT CCGACCCCGT GGGCACAATC GACCCCAATG ATATTCAAGG
XM_002494574    GCATTAGGTA CTGATAAAGT TGGTGATATT GCAGATGAAG ACGTGGAAGG
       YMR226C  GCTCTTGGCA GTGACCGTGT GGGCCAGATC GCAACGGAGG ATATCCAGGA
NM_001018495    GCTCTAGGTA CCGATAAAGT CATTGATCTT AATATTGATG ACGCCGTTAC
     XM_501554  GTCCACGGCA CCGAAAAGGT TGGCTCCATC AACCAGAACG ACATTGAGAT
     XM_957621  GTAAAAGG.. .....A...GC TCCCTCCATC GCCGAAGAAG ACATCAACGT
```

FIG. 12I

```
                451                                                 500
AABY01000127 TGTGTTTGAC ACCAACGTCA CAGCTTTAAT TAATATCACT CAAGCTGTGC
AACH01000019 CGTGTTCGAC ACCAACGTCT CGGCTTTGAT TAATGTCACA CAGGCTGTTC
AACA01000631 CGTGTTCGAC ACCAACGTCA CGGCGTTGAT CAACGTCACG CAAGCAGTGC
AACF01000116 AATGATGGAT ACCAATGTCT TGGGGTTGAT CAATGTGACG CAAGCTGTGT
      AE016819 GGTTTTCAAC ACTAATGTTA TCGGCTTGGT GCACTTGACT CAGGAGGTTC
       CR382139 GATGTTTCAA ACTAATGTTT TAGGGTTAAT TACCATCACA CAGGCTGTTT
XM_001387479 CATGATGCAA ACGAATGTCT TGGGACTCAT CAACATGACT CAGGCTGTGA
XM_001482184 CATGTTCCAT ACCAATGTTC TTGGGTTGAT AAACTTAACT CAGGAGGTGT
XM_002419771 AATGTTTGAT ACTAATGTCA TTGGAGTCAT TCGTATGACT CAAGCAGTTT
       CR380959 AATGGTTAAC ACCAACGTCC TTGCATTGAT CAATTTAACA CAATTGTTGT
XM_001645671 TATGTTTGAG ACTAACGTTT TTGGATTAAT CTGTTTAACA CAAGCTGTAC
  SAKL0H04730 AATGATCAAC ACCAATGTCT TAGCTCTTAT CAATATTACT CAAGCTGTCT
      XM_451902 TATGATAGAT ACTAACGTTG TTGCCCTTAT CAACGTTACC CAAGCTGTTT
XM_002553230 CATGATCCAG ACTAACGTTA TCGGGCTTAT AAATGTTACC CAAGCCGTTC
XM_002494574 TATGTTCGAC ACCAATGTCT TGGGGTTAAT CAAAGTTACT CAAGCTGTTT
       YMR226C CGTGTTTGAC ACCAACGTCA CGGCTTTAAT CAATATCACA CAAGCTGTAC
NM_001018495 CATGATTACT ACCAATGTTC TTGGTATGAT GGCTATGACT CGTGCGGTTC
      XM_501554 CATGTTCCAC ACAAACGTGC TCGGACTCAT TTCTGTCACT CAGCAGTTTG
      XM_957621 CATGTTTGCC ACCAACGTCA CCGGCCTGAT CAACATGACC CAAGCCATCC
```

FIG. 12J

```
                501                                                       550
AABY01000127 TGCCCATTTT TCAAGCCAAG AACTCAG... ..........G GGATATTGTG
AACH01000019 TGCCCATCTT CCAAGCTAAG AACTCAG... ..........G GGATATTGTG
AACA01000631 TGCCAATTTT CCAAGCCAAG AACTCAG... ..........G GGACATCGTC
AACF01000116 TGCACATTTT CCAAAGAAG AACTCCG... ..........G TGATATTGTG
     AE016819 TACCTATTAT GAAAGCCAAG AATTCCG... ..........G GGACATTGTC
     CR382139 TGCCAATCAT GAAAAAGAAG AACAGCG... ..........G AGATGTTGTT
XM_001387479 TTCCCATTTT TAAGGCTAAA AATTCTG... ..........G AGATATCGTC
XM_001482184 TACCCATCTT CAAAAAGAAA AATGCTG... ..........G AGATATTGTG
XM_002419771 TACCTTTACT TAAAAAAAAA CCTTATG... ..........C TGATGTGGTT
     CR380959 TGCCATTATT CAAGAAGAAG AACAGTG... ..........G TGATATCGTC
XM_001645671 TTCCAATATT CAAGGCTAAA AATGGTG... ..........G TGATATTGTC
  SAKL0H04730 TGCCAATCTT CAAAGCCAAG AATTTCG... ..........G TGATATCGTA
   XM_451902 TGCCTATTTT CAAAGCAAAG AATTCCG... ..........G TGACATCGTT
XM_002553230 TGCCCATCTT CAAGGCCAAA AACTCTG... ..........G TGATATCGTG
XM_002494574 TACCTATCTT CAAAAGAAAA AATTCTG... ..........G TGATGTCGTT
     YMR226C TGCCCATATT CCAAGCCAAG AATTCAG... ..........G AGATATTGTA
NM_001018495 TTCCTATATT CTACAGCAAA AACAAGG... ..........G TGATATTTTG
   XM_501554 TCGGCGAGAT GCGAAAGCGA AACAAGG... ..........G CGACATTGTC
   XM_957621 TGCCCATCTT CAAGGCCCGC GGCTCCGAGG GTGGTTCGGG AGACATTGTG
```

FIG. 12K

```
                551                                                       600
AABY01000127 AACTTGGG.T TCGGTGGCTG GCAGGGATGC ATACCCAACG GGTTCCATCT
AACH01000019 AACTTGGG.C TCGGTAGCTG GCAGAGATGC ATACCCAACG GGCTCCATCT
AACA01000631 AACTTGGGGC TCGGTGGCCG GCAGAGACGC ATACCCCACA GGCTCCATCT
AACF01000116 AACTTAGG.T TCAGTTGCTG GAAGAGATGC ATACCCAACA GGGTCCATTT
      AE016819 AATGTTGG.G TCGATTGCCG GCCGCGAAGC CTACCCTGGT GGCTCTATTT
       CR382139 AATATAGG.T TCAATTGCTG GAAGAGACTC TTACCCTGGA GGTGGAATTT
XM_001387479 AACATCGG.T TCGATTGCTG GAAGAGACCC TTACCCTGGT GGATCGATCT
XM_001482184 AACATTGG.C TCAGTGGCCG GTAGAGAACC TTACCCTGGA GGTGCAGTAT
XM_002419771 TTCATTGG.A AGTATTGCTG GACGTGTTCC TTATAAAAAT GGAGGTGGTT
       CR380959 AACTTGGG.A TCGATTGCTG GTAGAGACGC ATACCCAACG GGTGCTATAT
XM_001645671 AATTTAGG.G TCAATTGCAG GCATAGAAGC TTACCCAACA GGATCTATAT
  SAKL0H04730 AACTTGGG.G TCTGTCGCTG GTAGAGATGC TTATCCAACC GGTGCAATCT
      XM_451902 AACTTAGG.T TCTGTTGCCG GTAGAGATGC ATATCCAACT GGTTCTATCT
XM_002553230 AACCTGGG.T TCTGTCGCTG GCAGAGAAGC TTACCCTACA GGATCTATTT
XM_002494574 AACATTAG.T TCGGTTGCTG GTAGAGAGGC ATACCCAGGT GGTTCCATTT
       YMR226C AATTTGGG.T TCAATCGCTG GCAGAGACGC ATACCCAACA GGTTCTATCT
NM_001018495 AACGTTGG.C AGTATTGCCG GCAGAGAATC ATACGTAGGC GGCTCCGTTT
      XM_501554 AACATTGG.C TCCATCGCCG GACGAGAGCC CTACGTTGGA GGAGGAATCT
      XM_957621 AACATCGG.T TCTATTGCCG GGAGAGAACC ATACGCGGGA GGCTCCATCT
```

FIG. 12L

```
                601                                                   650
AABY01000127 ATTGTGCCTC CAAGTTTGCC GTGGGGGCGT TCACTGATAG TTTAAGAAAG
AACH01000019 ATTGTGCATC TAAGTTTGCC GTCGGGGCTT TCACTGAGAG TTTGAGAATG
AACA01000631 ACTGTGCTTC CAAGTTTGCC GTCGGTGCGT TCACTGACAG TTTGAGAAAG
AACF01000116 ACTGTGCTTC TAAATTTGCC GTGAGGGCCT TTACTGAAAG TTTGAGAAGG
     AE016819 ACTGTGCCAC GAAACATGCG GTCAAGGCTT TCACCAGGGC CATGCGGAAG
      CR382139 ACTGTCCAAC TAAGGCAAGT GTCAAGTCGT TTTCGCAAGT TTTAAGAAAG
XM_001387479 ACTGTGCCTC CAAGGCTGCT GTTAAGTTCT TCTCGCATTC TTTGAGAAAG
XM_001482184 ACTGTGCTTC AAAGGCAGCA GTTAACTACT TTTCTCATTC TTTGAGAAAG
XM_002419771 ATTGTGCATC TAAAGCTGCT GTTCGTAGTT TCACCGATAC ATTTAGAAAA
      CR380959 ACTGTGCAAC AAAACATGCT GTCAGGGCAT TCACACAATC CTTAAGGAAG
XM_001645671 ATTGTGCAAC TAAATTTGCA GTTAAAGCAT TCACTGAAAG TTTAAGAAAG
  SAKL0H04730 ACTGTGCTAG CAAACATGCA GTCAGAGCCT TCACTCAAAG TTTGAGGAAG
     XM_451902 ATTGTGCTTC GAAGCATGCT GTCAGAGCGT TCACTCAGTC TTTGAGAAAA
XM_002553230 ACTGCGCTAC GAAGCACGCG GTGCGTGCTT TCACCCAGAG CCTGCGCAAG
XM_002494574 ACTGTGCTAC TAAACACGCT GTTAAGGCAT TCACTGAAAG TTTGCGTAAG
       YMR226C ATTGTTGCCC TAAGTTTGCC GTGGGGGCGT TCACTGATAG TTTGAGAAAG
NM_001018495 ACTGCTCTAC CAAGTCTGCC CTTGCTCAAT TCACTTCCGC TTTGCGTAAG
     XM_501554 ACTGTGCCAC CAAGGCCGCC GTGCGATCTT TCACTGAGAC TCTCCGAAAA
     XM_957621 ACTGCGCCAC CAAGGCTGCT GTGCGCAGCT TCACCGACGC GCTGCGCAAG
```

FIG. 12M

```
                651                                                   700
AABY01000127 GAGCTTATCA ACACCAAGAT CAGAGTCATC CTAATCGCAC CAGGGCTAGT
AACH01000019 GAACTTATAA ACACTAAGAT TAGAGTCATT CTAATTGCAC CAGGGTTAGT
AACA01000631 GAACTGATCA ACACGAAGAT CAGAGTTATC TTGATCGCGC CGGGGCTGGT
AACF01000116 GAATTAATTA ATACCAAGAT TAGGGTGATA TTGATAGCCC CGGGTATCGT
      AE016819 GAGCTCATTA GCACCAAGAT CCGGGTCTTC GAAATTGCGC CGGGCTCTGT
        CR382139 GAATTGATTA GCACCAAGAT TAGAGTTCTT GAGGTTGACC CTGGTAATGT
XM_001387479 GAACTCATTA ACACCAGAAT CAGAGTTTTG GAAGTTGATC CAGGTGCTGT
XM_001482184 GAAACTATCA ATTCCAAAAT CAGGGTCATG GAGGTGGATC CTGGGGCAGT
XM_002419771 GAAACTATTA ATACTGGTAT TAGAGTCATT GAAGTTGATC CAGGTGCAGT
        CR380959 GAATTGATCA ACACCGACAT TAGAGTAATT GAAATTGCTC CTGGTATGGT
XM_001645671 GAATTGATTA ATACAAAGAT CAGAGTTATT GAAATTGCAC CAGGTATGGT
   SAKL0H04730 GAATTGGTGA ACACCAATAT CAGAGTGATT GAAATTGCTC CGGGTAATGT
      XM_451902 GAATTAATCA ATACTGGTAT TAGGGTCATT GAGATTGCTC CAGGTAATGT
XM_002553230 GAACTGATCA ACACAAACAT CAGGGTTATT GAGGTCGCTC CAGGTAACGT
XM_002494574 GAATTAGTCG ATACAAAAAT CAGAGTCATG AGTATTGATC CTGGTAATGT
        YMR226C GAGCTCATCA ACACTAAAAT TAGAGTCATT CTAATTGCAC CAGGGCTAGT
NM_001018495 GAGACTATTG ACACTCGCAT TCGTATTATG GAGGTTGATC CTGGCTTGGT
      XM_501554 GAGAACATCG ACACTCGAAT CCGAGTCATT GAGGTTGATC CTGGAGCCGT
      XM_957621 GAGCTGATCG CCACGCGCAT CCGTGTCATG GAGATTGACC CTGGCCAGGT
```

FIG. 12N

```
                701                                                 750
AABY01000127 CGAAACTGAA TTTTCACTGG TTAGATACAG AGGCAACGAG GAGCAAGCCA
AACH01000019 CGAAACTGAG TTTTCCCTGG TTAGATACAG AGGTAACGAA GAACAAGCCA
AACA01000631 TGAGACCGAG TTCTCACTGG TCAGATACAG AGGTAATGAG GAACAAGCTA
AACF01000116 CGAAACTGAA TTCTCAGTTG TTAGATACAA GGGTGATAAT GAGCGTGCTA
      AE016819 AGAAACGGAA TTCTCCATGG TTCGTATGCG CGGTAACGAA GAGAATGCCA
        CR382139 TGAAACTGAA TTTTCAAATG TCAGATTCAA GGGCGATATG GAAAAGGCAA
XM_001387479 GTTGACCGAG TTCTCTTTGG TTCGTTTCCA CGGTGATCAG GGAGCTGCTG
XM_001482184 AGAGACAGAG TTCTCGTTGG TTCGTTTTGG CGGTGATGCC GAGGCTGCGA
XM_002419771 ACTTACTGAA TTTAGTGTTG TTCGTTATAA AGGTGACACT GATGCTGCCG
        CR380959 CGAAACCGAG TTTTCTGTGG TCAGGTACAA AGGTGACAAG TCCAAAGCAG
XM_001645671 TAACACTGAA TTTTCTGTAA TTAGATATAA AGGTGACCAA GAAAGGCAG
  SAKL0H04730 TGAAACCGAG TTTTCCTTAG TTAGATATAA AGGTGATACG GACCGTGCTA
    XM_451902 CGAAACTGAG TTCTCTCTAG TTAGATACAA GGGTGATGCC GATCGTGCTA
XM_002553230 GGAGACCGAG TTTTCTCTGG TTAGATACAA GGGCGACTCT GAGAAAGCCA
XM_002494574 AGAGACCGAA TTTTCTATGG TTAGATTCCG TGGTGATACA GAAAAGGCAA
      YMR226C CGAGACTGAA TTTTCACTAG TTAGATACAG AGGTAACGAG GAACAAGCCA
NM_001018495 CGAAACCGAA TTCAGCGTTG TGAGATTCCA CGGAGACAAA CAAAAGGCTG
    XM_501554 TGAGACCGAG TTCTCCGTCG TGCGATTCCG AGGAGACAAG TCCAAGGCCG
    XM_957621 CGAGACCGAG TTCAGCGTGG TGAGGTTCTA TGGTGATAAG AACAAGGCTG
```

FIG. 12O

```
                751                                                 800
AABY01000127 AGAATGTCTA CAA.GGACAC CACCCCATTA ATGGCTGATG ACGTGGCTGA
AACH01000019 AGAATGTTTA CAA.GGACAC CACTCCGTTG ATGGCCGATG ACGTGGCTGA
AACA01000631 AAAACGTCTA CAA.GGACAC TACGCCGTTG ATGGCCGACG ACGTGGCTGA
AACF01000116 AATCTGTCTA CGATGGAGTT CACCCCTTGG AAG.CAGACG ACGTAGCAGA
     AE016819 AGAAAGTGTA CCA.GGGATT TGAACCCCTA GATGGTGATG ATATCGCTGA
      CR382139 AGCTGGTTTA CGC.GGGTAC TGAACCATTA TTATCCGAAG ACGTAGCTGA
XM_001387479 ATGCTGTTTA TGA.AGGTAC CCAACCTTTG GATGCCCTCTG ATATCGCAGA
XM_001482184 AAAAGGTGTA TGA.GGGAAC CGAGCCTTTG GGCCCAGAAG ATATTGCGGA
XM_002419771 ATGCTGTTTA TAC.TGGTAC TGAACCATTA ACACCAGAAG ATGTTGCTGA
      CR380959 ACGACGTCTA CAG.AGGTAC AACACCACTA TATGCCGATG ATATCGCGGA
XM_001645671 ATAAAGTTTA TGA.AAACAC TACTCCTTTA TATGCAGATG ACATCGCTGA
  SAKL0H04730 AAAAGGTTTA TGA.AGGTAC TAACCCATTA TATGCAGATG ACATTGCAGA
     XM_451902 AACAGGTTTA CAA.AGGTAC TACTCCTCTA TATGCAGATG ACATTGCTGA
XM_002553230 AGAAGGTTTA CGA.AGGCAC ACAACCCCTT TACGCTGACG ATATCGCAGA
XM_002494574 AGAAGGTTTA CCA.AGACAC TGTCCCATTA TATGCAGATG ACATTGCAGA
       YMR226C AGAATGTTTA CAA.GGATAC TACCCCATTG ATGGCTGATG ACGTGGCTGA
NM_001018495 ATAATGTTTA CAA.AAATAG TGAGCCTTTG ACACCCGAAG ACATTGCTGA
     XM_501554 ACGCTGTTTA CGC.TGGAAC CGAGCCTCTG GTCGCTGACG ATATTGCCGA
     XM_957621 ATGCCGTCTA TGC.CGGTGT CGATCCCTTG ACGCCCGATG ATATCGCAGA
```

FIG. 12P

```
                801                                                 850
AABY01000127 TTTGATCGTG TACGCAACTT CCAGGAAACA AAACACTGTA ATTGCAGACA
AACH01000019 TTTGATTGTG TATGCGACTT CAAGGAAGCA GAACACTGTA ATTGCAGACA
AACA01000631 CTTAATCGTA TATTCCACTT CCAGAAAGCA GAACACCGTG GTTGCCGACA
AACF01000116 TTTAATTGTA TACACCACTT CAAGAAAACA GAACACAGTA ATTGCTGACA
      AE016819 TACAATTGTC TATGCCACAT CCAGAAGATC CAACACCGTA GTTGCAGAGA
       CR382139 GGTTGTCGTA TTCGGACTTA CAAGAAAGCA AAATACCGTT ATTGCTGAGA
XM_001387479 AGTTATCGTG TTTGGTATCA CCAGAAAGCA GAACACCGTC ATAGCCGAAA
XM_001482184 AATCATTGTG TTTGCTGTGT CGAGAAAAGC CAAAACTGTC ATTGCGGAAA
XM_002419771 AGTGGTTGTT TTTGCATCTT CAAGAAAACA AAATACCGTT ATTGCTGATA
       CR380959 TTTGATTGTG TACTCTACCA GCAGAAAGCC AAACATGGTG GTAGCAGATG
XM_001645671 TTTGATAGTT TACACCACTT CTAGAAAATC GAATACCGTT ATCGCTGATG
  SAKL0H04730 CCTTATTGTG TATGCTACTT CTAGAAAGCC TAATACTGTC ATTGCGGATG
    XM_451902 CTTGATCGTT TATGCCACTT CAAGAAAACC TAATACTGTC ATCGCTGATG
XM_002553230 CCTAATCGTT TACGCAACCT CGAGAAAACC AAACACCGTC ATCGCGGACG
XM_002494574 TTTAATCGTC TATGCAACCT CTAGAAAGCA AAACACTGTC ATTGCTGACA
      YMR226C TCTGATCGTC TATGCAACTT CCAGAAAACA AAATACTGTA ATTGCAGACA
NM_001018495 GGTGATTCTT TTTGCCCTCA CTCGCAGAGA AAACGTCGTT ATTGCCGATA
    XM_501554 GTTCATCACC TACACTCTCA CTCGACGAGA GAATGTCGTC ATTGCCGATA
    XM_957621 GATCGTGGTG TTCGTCGTAA CACGACGGGA GAACGTTGTT GTTGCTGATA
```

FIG. 12Q

	851				900
AABY01000127	CGCTAATCTT	TCCAACCAAC	CAAGCATCGC	CTCACCACAT	CTTCCGTGGA
AACH01000019	CACTAATCTT	TCCTACCAAC	CAAGCGTCAC	CTTACCATAT	CTTTCGCGGG
AACA01000631	CCCTGATCTT	CCCCACCAAC	CAAGCCTCGC	CCTACCACAT	CTTTCGCGGT
AACF01000116	CTTTGATATT	CCCAACCTCT	CAAGGTTCCG	CATTCCACGT	CCATCGCGAT
AE016819	TGGTCGTTTA	CCCATCCGCG	CAAGGTTCTC	TGTACGATAC	TCACCGCAAC
CR382139	CATTAGTCTT	TTCAACCAAT	CAAGCCAGCT	CATCTCACTT	ATACCGTGAA
XM_001387479	CCTTGGTATT	CCCAAGTCAC	CAGGCTTCTG	CCTCTCATGT	TTACAAGGCT
XM_001482184	CTTTGGTGTT	TCCTACCCAT	CAGGCTGGAG	CAGTTCATGT	TCATAGAGGG
XM_002419771	CTTTGATTTT	CCCAAATCAT	CAAGCTTCTC	CAGATCATGT	TTATAGAAAA
CR380959	TCCTGGTCTT	CCCAACACAC	CAGGCATCGG	CTTCGCACAT	CTACAGGGGC
XM_001645671	TTTTGGTATT	CCCAACATGC	CAAGCTTCTG	CATCCCATAT	CTATCGTGGA
SAKL0H04730	TTTTGGTTTT	TGCTTCAAAC	CAAGCATCCC	CTTACCATAT	CTATCGCGGT
XM_451902	TTTTGGTATT	TGCTTCCAAC	CAAGCATCTC	CTTACCACAT	TTACCGTGGC
XM_002553230	TTTTGGTTTT	CGCTTCGAAC	CAGGCTTCGC	CTTACCACAT	TTACCGTGGT
XM_002494574	CTTTGATCTT	CTCTTCTAAC	CAGGCATCAC	CATACCACCT	CTACAGAGGC
YMR226C	CTTTAATCTT	TCCAACAAAC	CAAGCGTCAC	CTCATCATAT	CTTCCGTGGA
NM_001018495	CACTTGTTTT	CCCATCCCAT	CAAGGTGGTG	CCAATCATGT	GTACAGAAAG
XM_501554	CTCTCATTTT	CCCCAACCAC	CAGGCTTCTC	CTACTCACGT	CTACCGAAAG
XM_957621	CGTTGGTCTT	CCCTAGCCAT	CAGGCTGGCG	CTGGTATTAT	GCACCGCAAG

FIG. 12R

```
               901                   921
AABY01000127   TGA....... ........... . (SEQ ID NO:41)
AACH01000019   TGA....... ........... . (SEQ ID NO:47)
AACA01000631   TAA....... ........... . (SEQ ID NO:43)
AACF01000116   TAA....... ........... . (SEQ ID NO:45)
    AE016819   TAA....... ........... . (SEQ ID NO:49)
    CR382139   AGCGATAAAT AA........ . (SEQ ID NO:53)
XM_001387479   CCTAAGTAG. ........... . (SEQ ID NO:55)
XM_001482184   CCGCTTGAGT GA........ . (SEQ ID NO:57)
XM_002419771   CCTAATTAA. ........... . (SEQ ID NO:61)
    CR380959   GACTAA.... ........... . (SEQ ID NO:51)
XM_001645671   TAA....... ........... . (SEQ ID NO:59)
 SAKL0H04730   GACTAA.... ........... . (SEQ ID NO:69)
   XM_451902   GAATAG.... ........... . (SEQ ID NO:67)
XM_002553230   TAG....... ........... . (SEQ ID NO:65)
XM_002494574   TCTCAAGACA AAACCAATTG A (SEQ ID NO:63)
     YMR226C   TAA....... ........... . (SEQ ID NO:24)
NM_001018495   CAAGCGTAG. ........... . (SEQ ID NO:73)
   XM_501554   AACTGA.... ........... . (SEQ ID NO:71)
   XM_957621   TCGACATGA. ........... . (SEQ ID NO:75)
```

FIG. 12S

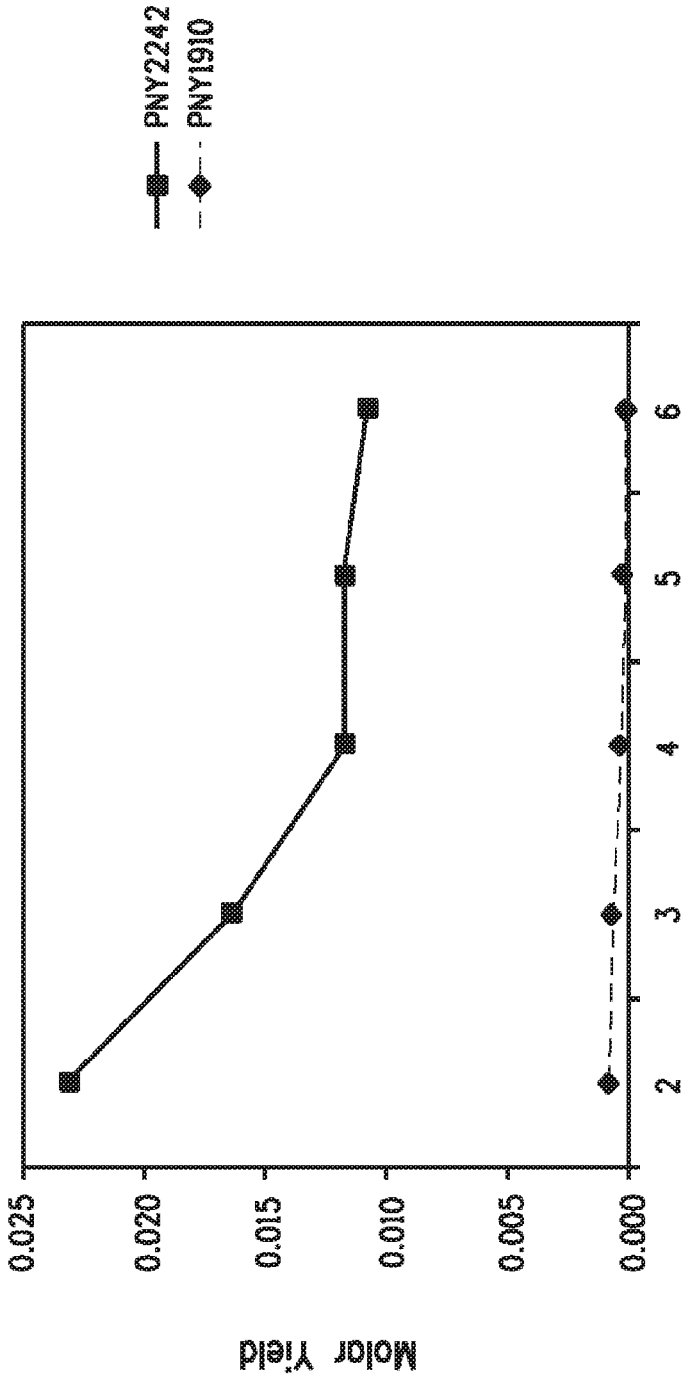

PNY2242
PNY1910
Molar Yield
0.025
0.020
0.015
0.010
0.005
0.000
2
3
4
5
6

FIG. 13

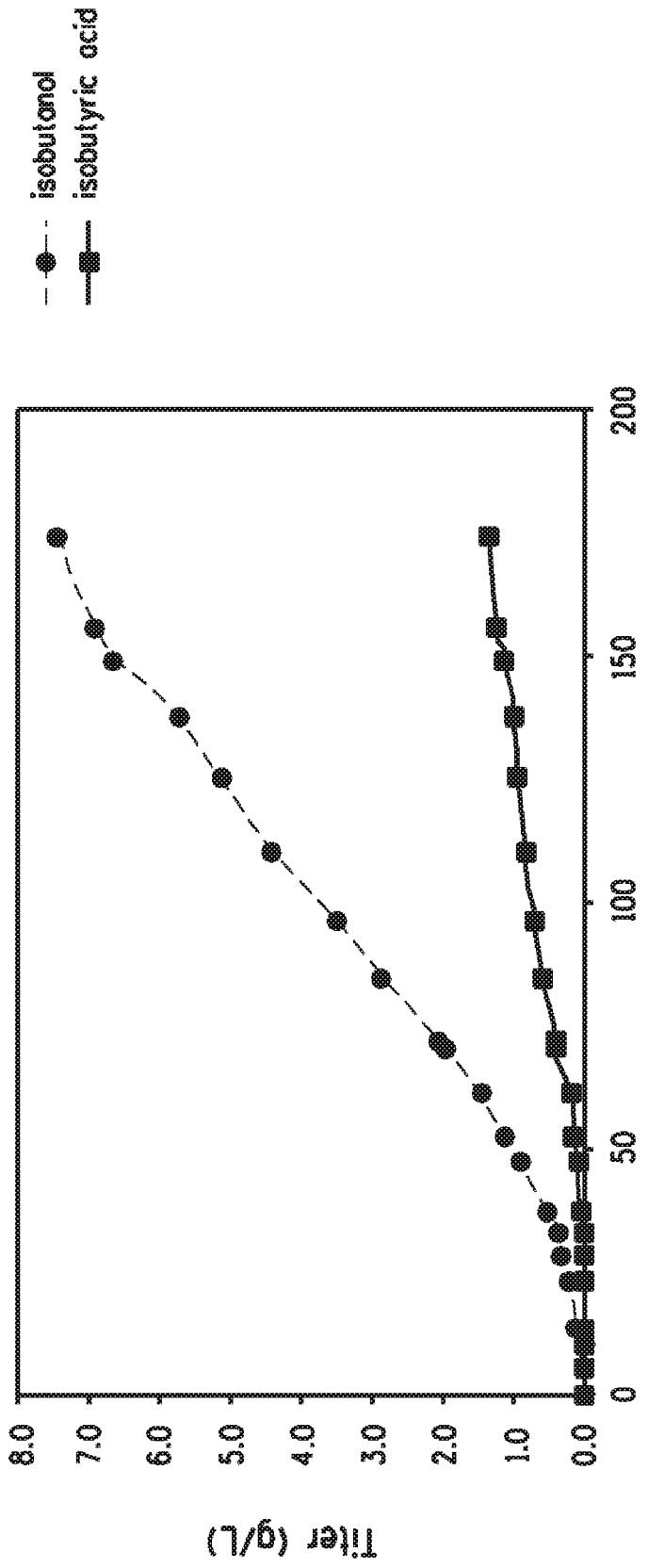

isobutanol
isobutyric acid
Titer (g/L)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
50
100
150
200
Time (hr)

FIG. 14

HOST CELLS AND METHODS FOR PRODUCTION OF ISOBUTANOL

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 13/428,585 (filed Mar. 23, 2012 which claims priority under 35 U.S.C. §119(e) to U.S. Application Nos. 61/472,484 (filed Apr. 6, 2011), 61/467,261 (filed Mar. 24, 2011), 61/472,487 (filed Apr. 6, 2011), 61/467,271 (filed Mar. 24, 2011), 61/570,513 (filed Dec. 14, 2011), 61/467,249 (filed Mar. 24, 2011), 61/472,497 (filed Apr. 6, 2011), and 61/472,474 (filed Apr. 6, 2011), each of which is in incorporated herein by reference in its entirety.

GOVERNMENT LICENSE RIGHTS

This invention was made with Government support under Agreement DE-AR0000006 awarded by the United States Department of Energy. The Government has certain rights in this invention.

FIELD OF THE INVENTION

The invention relates to recombinant host cells and methods for fermentative production of isobutanol.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY VIA EFS-WEB

The content of the electronically submitted Sequence Listing (Name: 20120323_CL5367USNA_SEQLIST.txt; Size 2,003,893 bytes; Date of Creation Mar. 23, 2012) is herein incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Butanol is an important industrial chemical, useful as a fuel additive, as a feedstock chemical in the plastics industry, and as a food grade extractant in the food and flavor industry. Each year 10 to 12 billion pounds of butanol are produced by petrochemical means and the need for this commodity chemical will likely increase in the future.

Methods for the chemical synthesis of isobutanol are known, such as oxo synthesis, catalytic hydrogenation of carbon monoxide (Ullmann's Encyclopedia of Industrial Chemistry, 6th edition, 2003, Wiley-VCH Verlag GmbH and Co., Weinheim, Germany, Vol. 5, pp. 716-719) and Guerbet condensation of methanol with n-propanol (Carlini et al., J. Molec. Catal. A. Chem. 220:215-220, 2004). These processes use starting materials derived from petrochemicals, are generally expensive, and are not environmentally friendly. The production of isobutanol from plant-derived raw materials would minimize green house gas emissions and would represent an advance in the art.

Isobutanol is produced biologically as a by-product of yeast fermentation. It is a component of "fusel oil" that forms as a result of the incomplete metabolism of amino acids by fungi. Isobutanol is specifically produced from catabolism of L-valine. After the amine group of L-valine is harvested as a nitrogen source, the resulting α-keto acid is decarboxylated and reduced to isobutanol by enzymes of the so-called Ehrlich pathway (Dickinson et al., J. Biol. Chem. 273:25752-25756, 1998).

Improvements and alternatives for the biosynthesis of butanol directly from sugars would improve economic viability and would represent an advance in the art.

SUMMARY OF THE INVENTION

Provided herein are recombinant yeast host cells and methods for the production of isobutanol.

In some embodiments, a recombinant host cell comprises an engineered isobutanol production pathway and (a) at least one of (i) a heterologous polypeptide with ketol-acid reductoisomerase (KARI) activity selected from the group consisting of (1) a polypeptide having at least about 90% identity to a KARI enzyme derived from *Bifidobacterium angulatum, Bifidobacterium dentium, Zyrnomonas mobilis, Clostridium beijerinckii* or *Anaerostipes caccae*, or an active fragment thereof (2) a polypeptide having at least about 90% identity or at least about 95% identity to SEQ ID NO: 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, or 65; or (ii) a heterologous polynucleotide encoding the heterologous polypeptide with KARI activity of (a); and (b) at least one host cell modification that enhances performance of the engineered isobutanol production pathway. In some embodiments, the combination of (a) and (b) results is a synergistic increase in isobutanol production pathway performance.

In some embodiments, a recombinant host cell comprises an isobutanol biosynthetic pathway and (a) a heterologous polypeptide with ketol-acid reductoisomerase (KARI) activity selected from the group consisting of (i) a polypeptide having at least about 90% identity to a KARI enzyme derived from *Bifidobacterium angulatum, Bifidobacterium dentium, Zymomonas mobilis, Clostridium beijerinckii* or *Anaerostipes caccae*, or an active fragment thereof, (ii) a polypeptide having at least about 90% identity or at least about 95% identity to SEQ ID NO: 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, or 65, (iii) a polypeptide having at least about 90% identity or at least about 95% identity to a KARI enzyme derived from *Bifidobacterium angulatum, Bifidobacterium dentium, Enterococcus gallinarum, Streptococcus thermophiles, Zymomonas mobilis, Clostridium beijerinckii, Anaerostipes caccae*, or *Lactococcus lactis* subsp. *cremoris* MG1363 or an active fragment thereof, wherein the polypeptide has a K_M for NADH less than about 50, (iv) a polypeptide having at least about 90% identity or at least about 95% identity to a KARI enzyme derived from *Staphylococcus capitis* SK14, *Staphylococcus epidermidis* M23864-W1, *Staphylococcus hominis* SK119, *Staphylococcus aureus* subsp. *aureus* TCH130, *Staphylococcus warneri* L37603, *Staphylococcus epidermidis* W23144, *Staphylococcus saprophyticus* subsp. *Saprophyticus* ATCC15305, *Staphylococcus carnosus* subsp. *Carnosus* TM300, *Listeria monocytogenes* EGO-e, *Listeria grayi* DSM 20601, *Enterococcus casseliflavus* EC30, *Enterococcus gallinarum* EG2, *Macrococcus caseolyticus* JCSC5402, *Streptococcus vestibularis, Streptococcus mutans* UA159, *Streptococcus gordonii* str, *cgakkus* sybstr. CH1, *Streptococcus suis* 89/1591, *Streptococcus infantarius* subsp. *infantarius* ATCC BAA-102, *Lactococcus lactis* subsp *cremoris* MG1363, *Lactococcus lactis, Leuconostoc mesenteroides* subsp *mesenteroides* ATCC8293, *Lactobacillus buchneri* ATCC 11577, *Staphylococcus haemolyticus* JCSC1435, *Staphylococcus epidermidis* ATCC12228, *Streptococcus pneumoniae* CGSP14, *Streptococcus pneumoniae* TIGR4, *Streptococcus sanguinis* SK36, *Streptococcus salivarius* SK126, *Streptococcus thermophilus* LMD-9, *Streptococcus pneumoniae* CCRI 1974M2, *Lactococcus lactis* subsp. *lactis* II1403, *Leuconostoc mesenteroides* subsp *cremoris* ATCC19254,

Leuconostoc mesenteroides subsp *cremoris, Lactobacillus brevis* subsp. *gravesensis* ATCC27305, or *Lactococcus lactis* subsp *lactis* NCDO2118 or an active fragment thereof, wherein the heterologous polypeptide has a K_M for NADH less than about 50, (v) a heterologous polypeptide with KARI activity having at least about 90% identity or at least about 95% identity to SEQ ID NO: 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, or 135 or an active fragment thereof, wherein the heterologous polypeptide has a K_M for NADH less than about 50; (b) a heterologous polynucleotide encoding the heterologous polypeptide with KARI activity of (a); (c) reduced or eliminated aldehyde dehydrogenase activity; (d) reduced or eliminated aldehyde oxidase activity; (e) reduced or eliminated acetolactate reductase activity; or (f) a combination thereof.

In one embodiment, the recombinant host cell comprises reduced or eliminated aldehyde dehydrogenase expression activity and reduced or eliminated acetolactate reductase expression or activity.

In another embodiment, the recombinant host cell comprises (i) reduced or eliminated aldehyde dehydrogenase expression or activity or reduced or eliminated acetolactate reductase expression or activity and (ii) a heterologous polynucleotide encoding a polypeptide having KARI activity and K_M for NADH less than 300 μM.

In another embodiment, the recombinant host comprises a heterologous polypeptide with KARI activity that has at least about 90% or at least about 95% identity to SEQ ID NO: 27, 29, 141, 143, 275, or 277.

In some embodiments, the recombinant host cell comprises a heterologous polypeptide with KARI activity that comprises substitutions in amino acids corresponding to S56 and S58 of SEQ ID NO: 27. In some embodiments, the polypeptide with KARI activity further comprises a substitution of one or more of the amino acids corresponding to I86, N87, T131, or T191 of SEQ ID NO: 27. In some embodiments, the polypeptide with KARI activity having at least 90% identity or at least 95% identity to SEQ ID NO: 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, or 65.

In some embodiments, the recombinant host cell has an effective isobutanol productivity of at least about 3, at least about 4, or at least about 5 grams per gram of cells after about 48 hours, wherein at least the last about 24 of the 48 hours are under anaerobic conditions.

In some embodiments, the recombinant host cell comprises a heterologous polypeptide with KARI activity that has a K_M for NADH less than about 350, less than about 100, less than about 50, or less than about 10 μM at pH 6.8.

In some embodiments, the recombinant host cell comprises a heterologous polypeptide with KARI activity that has at least about 90% identity or at least about 95% identity to SEQ ID NO: 376, 382, 378, or 275.

In some embodiments, the recombinant host cell comprises a heterologous polypeptide with KARI activity comprises an amino acid substitution at one or more of the positions corresponding to amino acids A41, S56, S58, I87, T131, T191, R227, or Q246 of a KARI enzyme derived from *Anaerostipes caccae* (SEQ ID NO:27).

In some embodiments, the recombinant host cell comprises a heterologous polypeptide with KARI activity that comprises SEQ ID NO: 33 or SEQ ID NO:35 or an active fragment thereof.

In another embodiment, the recombinant host cell comprises a deletion, mutation, and/or substitution in an endogenous polynucleotide encoding a polypeptide having aldehyde dehydrogenase activity. In some embodiments, the polypeptide having aldehyde dehydrogenase activity catalyzes the conversion of isobutyraldehyde to isobutyric acid. In some embodiments, the polypeptide having aldehyde dehydrogenase activity corresponds to Enzyme Commission Number EC 1.21.3, EC 1.2.1.4, and/or EC1.2.1.5. In some embodiments, the host cell is *S. cerevisiae* and the polypeptide having aldehyde dehydrogenase activity is ALD2, ALD3, ALD4, ALD5, ALD6 or a homolog thereof. In some embodiments, the host cell is *K. lactis* and the polypeptide having aldehyde dehydrogenase activity is KLLA0F00440, KLLA0E23057, KLLA0D10021, or KLLA0D09999G. In some embodiments, the host cell is *P. stipitis* and the polypeptide having aldehyde dehydrogenase activity is ALD2, ALD3, ALD4, ALD5, or ALD7. In some embodiments, the host cell is *Lactobacillus plantarum* and said polypeptide having aldehyde dehydrogenase activity is AldH. In some embodiments, the host cell is *E. coli* and the polypeptide having aldehyde dehydrogenase activity is aldA, aldB, or aldH.

In another embodiment, the host cell comprises a deletion, mutation, and/or substitution in an endogenous polynucleotide encoding a polypeptide having aldehyde oxidase activity. In some embodiments, the polypeptide having aldehyde oxidase activity catalyzes the conversion of isobutyraldehyde to isobutyric acid. In some embodiments, the polypeptide having aldehyde oxidase activity corresponds to Enzyme Commission Number EC 1.2.3.1. In some embodiments, the polypeptide having aldehyde oxidase activity is AOX1 and/or AOX2.

In another embodiment, the host cell comprises a deletion, mutation, and/or substitution in an endogenous polynucleotide encoding a polypeptide having acetolactate reductase activity. In some embodiments, the polypeptide having acetolactate reductase activity comprises a polypeptide encoded by a polynucleotide selected from the group consisting of SEQ ID NO: 676, SEQ ID NO: 678, SEQ ID NO: 680, SEQ ID NO: 682, SEQ ID NO: 684, SEQ ID NO: 686, SEQ ID NO: 688, SEQ ID NO: 690, SEQ ID NO: 692, SEQ ID NO: 694, SEQ ID NO: 696, SEQ ID NO:702, SEQ ID NO: 704, SEQ ID NO: 706, SEQ ID NO: 708, SEQ ID NO: 710, SEQ ID NO: 712, SEQ ID NO: 714, SEQ ID NO: 716, SEQ ID NO: 718, SEQ ID NO: 720, SEQ ID NO: 722, SEQ ID NO: 724, SEQ ID NO:726, SEQ ID NO:728, and SEQ ID NO: 730. In some embodiments, the polypeptide having acetolactate reductase activity is YMR226C.

In another embodiment, the recombinant host cell is a yeast host cell. In some embodiments, the yeast is selected from the group consisting of *Saccharomyces, Schizosaccharomyces, Hansenula, Candida, Kluyveromyces, Yarrowia, Issatchenkia*, or *Pichia*. In some embodiments, the host cell is *Saccharomyces cerevisiae.*

In another embodiment, the host cell is a bacterial cell. In some embodiments, the bacterial cell is a *Clostridium, Zymomonas, Escherichia, Salmonella, Rhodococcus, Pseudomonas, Bacillus, Lactobacillus, Enterococcus, Pediococcus, Alcaligenes, Klebsiella, Paenibacillus, Arthrobacter, Corynebacterium, Brevibacterium, Lactococcus, Leuconostoc, Oenococcus, Pediococcus*, or *Streptococcus* cell. In some embodiment, the bacterial cell is not *E. coli.*

In some embodiments, the engineered isobutanol production pathway of the recombinant host cell comprises the following substrate to product conversions: (a) pyruvate to acetolactate, (b) acetolactate to 2,3-dihydroxyisovalerate, (c) 2,3-dihydroxyisovalerate to 2-ketoisovalerate, (d) 2-ketoisovalerate to isobutyraldehyde, and (e) isobutyraldehyde to isobutanol and more than one of the substrate to product conversions is catalyzed by an enzyme that is heterologous to the host cell. In some embodiments, all of the substrate to

product conversions are catalyzed by enzymes heterologous to the host cell. In some embodiments, at least one heterologous polynucleotide encoding an enzyme heterologous to the host cell is chromosomally integrated into the host cell. In some embodiments, the substrate to product conversions are catalyzed by enzymes substantially localized to the cytosol. In some embodiments, the substrate to product conversion for isobutyraldehyde to isobutanol is catalyzed by an alcohol dehydrogenase enzyme that utilizes NADH as a cofactor. In some embodiments, the conversion of acetolactate to 2,3-dihydroxyisovalerate is catalyzed by a KARI that can use NADH as a cofactor.

In some embodiments, the host cell comprises the plasmid pLH702 or pLH701 or a plasmid having the same coding regions. In some embodiments, the host cell comprises the plasmid pBP915 or a plasmid having the same coding regions. In some embodiments, the host cell comprises the plasmid pYZ067ΔkivDΔhADH or a plasmid having the same coding regions.

In some embodiments, the host cell comprises reduced, disrupted, or eliminated ability to convert acetolactate to 2,3-dihydroxy-2-methylbutyrate.

In some embodiments, the host cell is yeast and has reduced or eliminated pyruvate decarboxylase expression or activity. In some embodiments, the host cell has reduced or eliminated PDC1, PDC5, or PDC6 activity or a combination thereof.

In some embodiments, the host cell has reduced or eliminated NAD-dependent glycerol-3-phosphate dehydrogenase expression or activity. In some embodiments, the host cell has reduced GPD2 activity.

In some embodiments, the host cell has reduced or eliminated FRA2 expression or activity.

In some embodiments, the host cell produces isobutanol under anaerobic conditions and the molar ratio of isobutanol to glycerol is greater than 1.

In some embodiments, the polypeptide having ketol-acid reductoisomerase activity matches the profile HMM given provided in Table Z with a profile HMM E value of $<10^{-3}$.

In some embodiments, the host cell produces isobutanol at a yield greater than about 25%, about 50%, about 75%, or about 90% of theoretical yield.

In some embodiments, isobutanol and ethanol are produced.

Methods for producing isobutanol include methods comprising providing a recombinant host cell as described above and contacting the host cell with a carbon substrate under conditions whereby isobutanol is produced. In some embodiments, at least a portion of the contacting occurs under anaerobic conditions. In some embodiments, the contacting occurs in the presence of an extractant. In some embodiments, the contacting occurs in the presence of a sufficient quantity of organic extractant to form a two-phase system comprising an aqueous phase and an organic phase. In some embodiments, one or more of the effective rate, effective titer, or effective yield of isobutanol is increased as compared to methods using a recombinant host cell that does not comprise a heterologous polypeptide with KARI activity, a heterologous polynucleotide encoding a polypeptide with KARI activity, reduced or eliminated aldehyde dehydrogenase activity, reduced or eliminated aldehyde oxidase activity, reduced or eliminated acetolactate reductase activity, or a combination thereof. In some embodiments, one or more of the effective rate, effective titer, or effective yield of isobutanol is increased as compared to methods using a recombinant host cell that does not comprise (i) a heterologous polypeptide with KARI activity or a heterologous polynucleotide encoding a polypeptide with KARI activity and (ii) at least one modification that enhances performance of the engineered isobutanol production pathway. In some embodiments, DHMB production, isobutyric acid production, or both is reduced as compared to methods using a recombinant host cell that does not comprise a heterologous polypeptide with KARI activity, a heterologous polynucleotide encoding a polypeptide with KARI activity, reduced or eliminated aldehyde dehydrogenase activity, reduced or eliminated aldehyde oxidase activity, reduced or eliminated acetolactate reductase activity, or a combination thereof. In some embodiments, DHMB production, isobutyric acid production, or both is reduced as compared to methods using a recombinant host cell that does not comprise (i) a heterologous polypeptide with KARI activity or a heterologous polynucleotide encoding a polypeptide with KARI activity and (ii) at least one modification that enhances performance of the engineered isobutanol production pathway. In some embodiments, the molar ratio of isobutanol to glycerol is greater than 1.

Methods for producing isobutanol also comprise providing a recombinant host cell that produces isobutanol and contacting the host cell with a carbon substrate under conditions whereby isobutanol is produced, wherein at least a portion of the contacting occurs under anaerobic conditions, and wherein the ratio of isobutanol to glycerol produced is greater than 1.

Methods for producing isobutanol also comprise growing a recombinant yeast comprising a biosynthetic pathway capable of converting pyruvate to acetolactate under conditions whereby butanol is produced and removing DHMB from the culture.

Compositions produced by such methods are also provided herein. In some embodiments, the composition comprises isobutanol and a recombinant host cell provided above. In some embodiments, the composition comprises butanol and no more than about 0.5 mM DHMB.

Fermentative compositions are also provided herein. In some embodiments, a fermentative composition comprises the host cell and isobutanol produced according to the methods provided above.

Compositions comprising i) a recombinant yeast capable of producing butanol, ii) butanol, and iii) no more than about 0.5 mM DHMB are also provided.

Methods for producing a recombinant host cell are also provided. Such methods can comprise (a) providing a recombinant host cell comprising a modification in a polynucleotide encoding a polypeptide having aldehyde dehydrogenase activity or aldehyde oxidase activity; and (b) transforming the host cell with a polynucleotide encoding a polypeptide of an isobutanol biosynthetic pathway.

Methods for reducing or eliminating the conversion of isbutyraldehye to isobutyric acid are also provided. Such methods can comprise (a) providing the recombinant host cell as described herein; and (b) subjecting the host cell to conditions wherein the conversion of isbutyraldehye to isobutyric acid is reduced or eliminated compared to methods using a recombinant host cell that does not comprise reduced or eliminated aldehyde dehydrogenase and/or aldehyde oxidase activity.

Certain polypeptides are also provided herein. In some embodiments, the polypeptides comprise at least about 90% identity or at least about 95% identity or at least about 99% identity to SEQ ID NO: 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 417, 419, 421, 423, 425, 427, 429, 431, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466,

467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 511, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 525, 526, 527, 528, 529, 530, 531, 532, 533, 534, 535, 536, 537, 624, 626, 628, 630, 632 or an active fragment thereof and have ketol-acid reductoisomerase activity. In some embodiments, the polypeptides comprise at least about 90% identity or at least about 95% identity or at least about 99% identity to SEQ ID NO: 417, 419, 421, 423, 425, or 427 or an active fragment thereof and have ketol-acid reductoisomerase activity. In some embodiments, a polypeptide comprises a sequence with at least about 90% identity, at least about 95% identity, or at least about 99% identity to SEQ ID NO: 927, 928, 196, 266, 267, 389, 405, 637, 781, 782, 783, 835, 853, 854, 855, 856, 857, or 859.

Polynucleotides encoding such polypeptides and host cells comprising such polynucleotides and polypeptides are also provided.

Methods of converting acetolactate to 2,3-dihydroxyisovalerate are also provided. For example, such methods can comprise (a) providing a polypeptide described above, and (b) contacting the polypeptide with acetolactate under conditions wherein 2,3-dihydroxyisovalerate is produced.

Recombinant yeast cells are also provided herein. In some embodiments, a recombinant yeast comprises a biosynthetic pathway capable of converting pyruvate to acetolactate, and the yeast produces less than 0.01 moles 2,3-dihydroxy-2-methyl butyrate (DHMB) per mole of sugar consumed. In some embodiments, a recombinant yeast comprises capable of converting pyruvate to acetolactate, and the yeast produces DHMB at a rate of less than about 1.0 mM/hour. In some embodiments, a recombinant yeast comprises a biosynthetic pathway capable of converting pyruvate to acetolactate, and the yeast produces an amount of 2,3-dihydroxy-3-isovalerate (DHIV) that is at least about 1.5 times the amount of DHMB produced.

Methods of identifying a gene involved in DHMB production are also provided. In some embodiments, the methods comprise (i) providing a collection of yeast strains comprising at least two or more gene deletions; (ii) measuring the amount of DHMB produced by individual yeast strains; (iii) selecting a yeast strain that produces no more than about 1.0 mM DHMB/hour; and (iv) identifying the gene that is deleted in the selected yeast strain. In some embodiments, the methods comprise (i) providing a collection of yeast strains that over-express at least two or more genes; (ii) measuring the amount of DHMB produced by individual yeast strains; (iii) selecting a yeast strain that produces at least about 1.0 mM DHMB; and (iv) identifying the gene that is over-expressed in the selected yeast strain.

Methods for the production of butanol are also provided. In some embodiments, the methods comprise (a) growing a recombinant yeast comprising a biosynthetic pathway capable of converting pyruvate to acetolactate under conditions whereby butanol is produced; and b) measuring DHIV and/or DHMB concentration. In some embodiments, the growing and measuring can be performed simultaneously or sequentially and in any order. In some embodiments, the measuring comprises liquid chromatography-mass spectrometry.

Methods for increasing ketol-acid reductoisomerase (KARI) activity are also provided. In some embodiments, the methods comprise (a) providing a composition comprising acetolactate, a KARI enzyme, and an acetolactate reductase enzyme and (b) decreasing DHMB levels. In some embodiments, decreasing DHMB levels is achieved by decreasing acetolactate reductase enzyme activity. In some embodiments, decreasing DHMB levels is achieved by removing DHMB from the composition.

In some embodiments, increasing KARI enzyme productivity in a host cell can comprises culturing a host cell, wherein the host cell comprises a heterologous KARI enzyme and at least one genetic modification that reduces, disrupts, or eliminates acetolactate reductase expression or activity, and wherein the KARI enzyme activity is decreased in the presence of DHMB. In some embodiments, the KARI has at least about 90%, at least about 95%, or at least about 99% identity to *E. coli* or *L. lactis* KARI. In some embodiments, the reduced, disrupted, or eliminated acetolactate reductase expression or activity substantially reduces the presence of DHMB.

Methods for increasing dihydroxyacid dehydratase (DHAD) activity are also provided. In some embodiments, the methods comprise (a) providing a composition comprising dihydroxyisovalerate (DHIV) and a DHAD enzyme and (b) decreasing DHMB levels.

BRIEF DESCRIPTION OF THE FIGURES AND INCORPORATION BY REFERENCE OF THE TABLE FILED ELECTRONICALLY HEREWITH

The invention can be more fully understood from the following detailed description, the Figures, and the accompanying sequence descriptions, which form part of this application.

FIG. **1**—Shows four different isobutanol biosynthetic pathways. The steps labeled "a", "b", "c", "d", "e", "f", "g", "h" "i" "j" and "k" represent the substrate to product conversions described below.

FIG. **2** depicts an alignment of the amino acid sequences of the KARI from *Pseudomonas fluorescens* ("PF5"; SEQ ID NO: 5) and KARI from *Anaerostipes caccae* ("K9"; SEQ ID NO: 27). The bolded positions are targeted for mutagenesis as described herein.

FIGS. **3**A, **3**B and **3**C depict an alignment of the amino acid sequences of KARI enzymes from *Bifidobacterium angulatum* DSM 20098 ("K1"; SEQ ID NO: 141), *Bifidobacterium dentium* ATCC 27678 ("K2"; SEQ ID NO: 143), *Clostridium beijerinckii* NCIMB 8052 ("K7"; SEQ ID NO: 275), *Anaerostipes caccae* DSM 14662 ("K9"; SEQ ID NO: 27), *Enterococcus gallinarum* EG2 ("K25" SEQ ID NO: 376), *Streptococcus thermophilus* LIVID-9 ("K26" SEQ ID NO: 121), *Lactococcus lactis* subsp. *cremoris* MG1363 ("K29"; SEQ ID NO: 382), *Zymomonas mobilis* ("S2"; SEQ ID NO: 277), and *Lactococcus lactis* ("LTS"; SEQ ID NO: 380). The bolded positions are targeted for mutagenesis as described herein.

FIG. **4** is a plasmid map of pLH556 (pHR81-Pll5-Pf5.KARI) vector (SEQ ID NO: 138).

FIG. **5** shows the specific rate of isobutanol production, Qp, of the two strains, PNY1910 and PNY2242.

FIG. **6** shows the accumulation of DHIV+DHMB in the culture supernatant during the fermentation time course with PNY1910 (triangles) and PNY2242 (diamonds). (DHMB and DHIV are not distinguished by the HPLC method used.)

FIG. **7** shows the yield of glycerol, pyruvic acid, 2,3-butanediol (BDO), DHIV/DHMB, α-ketoisovalerate (aKIV), and isobutyric acid (iBuAc), DHIV and DHMB are shown together as these are not distinguished by the HPLC method used.

FIG. **8** shows a summary of V_{max}/K_M values for K9G9 variants as described in Example 16.

FIGS. **9**A, B, and C show isobutanol to glycerol molar yield ratios, isobutanol molar yields, and isobutanol titers for K9 variants as described in Example 19.

FIG. **10** shows an isobutanol biosynthetic pathway. Step "a" represents the conversion of pyruvate to acetolactate. Step "b" represents the conversion of acetolactate to DHIV. Step "c" represents the conversion of DHIV to KIV. Step "d" represents the conversion of KIV to isobutyraldehyde. Step "e" represents the conversion of isobutyraldehyde to isobutanol. Step "f" represents the conversion of acetolactate to DHMB.

FIG. **11** shows a phylogenetic tree of YMR226C homologs from species of ascomycete yeast. A filamentous fungi (*Neurospora crassa*) sequence is included as an outgroup.

FIG. **12** shows a multiple sequence alignment (MSF Format) of nucleotide sequences of ORFs with homology to YMR226C. The gene names shown correspond to the accession numbers and SEQ ID NOs. given in Table 7. The alignment was produced by AlignX (Vector NTI).

FIG. **13** shows a graph of the molar yield of DHMB over time.

FIG. **14** depicts the production of isobutanol and isobutyric acid in yeast strain NYLA84.

Table Z—is a table (filed electronically herewith and incorporated by reference) of the Profile HMM of experimentally verified KARI enzymes listed in Table 1 and as described in US App. Pub. Nos. 2010/0197519 and 2009/0163376, which are herein incorporated by reference in their entireties.

TABLE 1

Experimentally verified KARI enzymes.

GI Number	Accession	SEQ ID NO:	Microorganism
70732562	YP_262325.1	5	*Pseudomonas fluorescens* Pf-5
15897495	NP_342100.1	1	*Sulfolobus solfataricus* P2
18313972	NP_560639.1	2	*Pyrobaculum aerophilum* str. IM2
76801743	YP_326751.1	7	*Natronomonas pharaonis* DSM 2160
16079881	NP_390707.1	8	*Bacillus subtilis* subsp. *subtilis* str. 168
19552493	NP_600495.1	9	*Corynebacterium glutamicum* ATCC 13032
6225553	O32414	10	*Phaeospirilum molischianum*
17546794	NP_520196.1	3	*Ralstonia solanacearum* GMI1000
56552037	YP_162876.1	11	*Zymomonas mobilis* subsp. *mobilis* ZM4
114319705	YP_741388.1	12	*Alkalilimnicola ehrlichei* MLHE-1
57240359	ZP_00368308.1	13	*Campylobacter lari* RM2100
120553816	YP_958167.1	14	*Marinobacter aquaeolei* VT8
71065099	YP_263826.1	15	*Psychrobacter arcticus* 273-4
83648555	YP_436990.1	16	*Hahella chejuensis* KCTC 2396
74318007	YP_315747.1	17	*Thiobacillus denitrificans* ATCC 25259
67159493	ZP_00420011.1	18	*Azotobacter vinelandii* AvOP
66044103	YP_233944.1	19	*Pseudomonas syrigae* pv. *syrigae* B728a
28868203	NP_790822.1	20	*Pseudomonas syrigae* pv. *tomato* str. DC3000
26991362	NP_746787.1	21	*Pseudomonas putida* KT2440
104783656	YP_610154.1	22	*Pseudomonas entomophila* L48
146306044	YP_001186509.1	23	*Pseudomonas mendocina* ymp
15599888	NP_253382.1	4	*Pseudomonas aeruginosa* PAO1
42780593	NP_977840.1	24	*Bacillus cereus* ATCC 10987
42781005	NP_978252.1	25	*Bacillus cereus* ATCC 10987
266346	Q01292	6	*Spinacia oleracea*

The eleven positions in the profile HMM representing the columns in the alignment which correspond to the eleven cofactor switching positions in *Pseudomonas fluorescens* Pf-5 KARI are identified as positions 24, 33, 47, 50, 52, 53, 61, 80, 115, 156, and 170. Table Z is submitted herewith electronically and is incorporated herein by reference.

The sequences provided in the sequence listing filed herewith (Name: 20120323_CL5367USNA_SEQLIST.txt; Size 2,003.893 bytes; Date of Creation Mar. 23, 2012), is herein incorporated by reference.

Consistent with the World Intellectual Property Organization (WIPO) Standard ST.25 (2009), certain primers given in the sequence listing and herein use N to represent nucleotides a or g or c or t. K is used to represent g or t. M is used to represent a or c.

DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In case of conflict, the present application including the definitions will control. Also, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. All publications, patents and other references mentioned herein are incorporated by reference in their entireties for all purposes as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference, unless only specific sections of patents or patent publications are indicated to be incorporated by reference.

Although methods and materials similar or equivalent to those disclosed herein can be used in practice or testing of the present invention, suitable methods and materials are disclosed below. The materials, methods and examples are illustrative only and are not intended to be limiting. Other features and advantages of the invention will be apparent from the detailed description and from the claims.

The last step in the biosynthesis of isobutanol via a pyruvate-utilizing biosynthetic pathway is the conversion of isobutyraldehyde to isobutanol (FIG. **1**). A side reaction in this pathway is the conversion of isobutyraldehyde to isobutyric acid which results in reduced amounts of isobutyraldehyde available to convert into isobutanol and reduced isobutanol yield. For an efficient biosynthetic process, there is a need to prevent the conversion of isobutyraldehyde to isobutyric acid such that increased amounts of isobutyraldehyde are available for conversion to isobutanol and isobutanol yields are increased.

Aldehyde dehydrogenases are a family of enzymes that catalyze the oxidation (dehydrogenation) of aldehydes (Wang et al., *J. Bacteriol.* 180:822-30, 1998; Navarro-Avino et al., *Yeast* 15:829-42, 1999; and Saint-Prix et al., *Microbiology* 150:2209-20, 2004). There is a need to identify suitable aldehyde dehydrogenases that can be modified to reduce or eliminate aldehyde dehydrogenase activity, and can reduce or eliminate the conversion of isobutyraldehyde to isobutyric acid, such that increased amounts of isobutyraldehyde are available for conversion to isobutanol and isobutanol yields are increased.

Aldehyde oxidases are a family of enzymes that catalyze the production of carboxylic acids from aldehydes (Nomura et al., *Biosci. Biotechnol. Biochem.* 62:1134-7, 1998; and Johnson et al., *Genetics* 151:1379-1391, 1999). There is a need to identify suitable aldehyde oxidases that can be modified to reduce or eliminate aldehyde oxidase activity and can reduce or eliminate the conversion of isobutyraldehyde to

isobutyric acid, such that increased amounts of isobutyraldehyde are available for conversion to isobutanol and isobutanol yields are increased.

The biosynthesis pathway for the production of butanol in genetically engineered yeast includes the conversion of acetolactate to 2,3-dihydroxy-3-isovalerate (DHIV), which is subsequently converted to butanol. See FIG. **10**. However, a side reaction in this pathway, which decreases the overall production of butanol, is the conversion of acetolactate to 2,3-dihydroxy-2-methylbutyrate (DHMB). In fact, Applicants have discovered that DHMB has inhibitory effects on enzymes (dihydroxyacid dehydratase and ketol-acid reductoisomerase) in an isobutanol production pathway. For an efficient biosynthetic process, there is a need to prevent the conversion of acetolactate to DHMB.

Applicants have solved the stated problems by providing recombinant yeast host cells comprising an isobutanol biosynthetic pathway; and at least one of: i) reduced or eliminated aldehyde dehydrogenase activity ii) reduced or eliminated aldehyde oxidase activity iii) reduced or eliminated acetolactate reductase activity; iv) a heterologous polynucleotide encoding a polypeptide having ketol-acid reductoisomerase activity; and v) a heterologous polypeptide having ketol-acid reductoisomerase activity. Further, Applicants provide methods of producing butanol utilizing such host cells. Such recombinant host cells can be used to increase the production of a product of a biosynthetic pathway (e.g., isobutanol, 1-butanol, or 2-butanol) and/or reduce or eliminate the conversion of pathway intermediates to undesirable byproducts. Applicants have also provided a suitable screening strategy for evaluating various candidate enzymes. The identified enzymes can be altered to enhance the production of a product of a biosynthetic pathway (e.g., isobutanol, 1-butanol, or 2-butanol) and/or reduce or eliminate the conversion of pathway intermediates to undesirable byproducts.

In order to further define this invention, the following terms, abbreviations and definitions are provided.

It will be understood that "derived from" with reference to polypeptides disclosed herein encompasses sequences synthesized based on the amino acid sequences of the KARIs present in the indicated organisms as well as those cloned directly from the organism's genetic material.

As used herein, the terms "comprises," "comprising," "includes," "including," "has," "having," "contains" or "containing," or any other variation thereof, will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers and are intended to be non-exclusive or open-ended. For example, a composition, a mixture, a process, a method, an article, or an apparatus that comprises a list of elements is not necessarily limited to only those elements but can include other elements no expressly listed or inherent to such composition, mixture, process, method, article, or apparatus. Further, unless expressly stated to the contrary, "or" refers to an inclusive or and not to an exclusive or. For example, a condition A or B is satisfied by any one of the following: A is true (or present) and B is false (or not present), A is false (or not present) and B is true (or present), and both A and B are true (or present).

As used herein, the term "consists of," or variations such as "consist of" or "consisting of," as used throughout the specification and claims, indicate the inclusion of any recited integer or group of integers, but that no additional integer or group of integers can be added to the specified method, structure, or composition.

As used herein, the term "consists essentially of," or variations such as "consist essentially of" or "consisting essentially of," as used throughout the specification and claims, indicate the inclusion of any recited integer or group of integers, and the optional inclusion of any recited integer or group of integers that do not materially change the basic or novel properties of the specified method, structure or composition. See M.P.E.P. §2111.03.

Also, the indefinite articles "a" and an preceding an element or component of the invention are intended to be nonrestrictive regarding the number of instances, i.e., occurrences of the element or component. Therefore "a" or an should be read to include one or at least one, and the singular word form of the element or component also includes the plural unless the number is obviously meant to be singular.

The term "invention" or "present invention" as used herein is a non-limiting term and is not intended to refer to any single embodiment of the particular invention but encompasses all possible embodiments as described in the claims as presented or as later amended and supplemented, or in the specification.

As used herein, the term "about" modifying the quantity of an ingredient or reactant of the invention employed refers to variation in the numerical quantity that can occur, for example, through typical measuring and liquid handling procedures used for making concentrates or solutions in the real world; through inadvertent error in these procedures; through differences in the manufacture, source, or purity of the ingredients employed to make the compositions or to carry out the methods; and the like. The term "about" also encompasses amounts that differ due to different equilibrium conditions for a composition resulting from a particular initial mixture. Whether or not modified by the term "about", the claims include equivalents to the quantities. In one embodiment, the term "about" means within 10% of the reported numerical value, or within 5% of the reported numerical value.

As used herein, "synergistic" refers to a greater-than-additive effect produced by a combination (i.e., an effect that is greater than the sum of individual effects) or an additive effect when the individual effects are not expected to be additive. The term also refers to the addition of one compound which results in less of another compound being required.

The term "butanol biosynthetic pathway" as used herein refers to the enzymatic pathway to produce 1-butanol, 2-butanol, or isobutanol. For example, isobutanol biosynthetic pathways are disclosed in U.S. Patent Application Publication No. 2007/0092957, which is incorporated by reference herein.

The term "isobutanol biosynthetic pathway" refers to the enzymatic pathway to produce isobutanol. Certain isobutanol biosynthetic pathways are illustrated in FIG. **1** and described herein. From time to time "isobutanol biosynthetic pathway" is used synonymously with "isobutanol production pathway".

The term "butanol" as used herein refers to 2-butanol, 1-butanol, isobutanol or mixtures thereof. Isobutanol is also known as 2-methyl-1-propanol.

A recombinant host cell comprising an "engineered alcohol production pathway" (such as an engineered butanol or isobutanol production pathway) refers to a host cell containing a modified pathway that produces alcohol in a manner different than that normally present in the host cell. Such differences include production of an alcohol not typically produced by the host cell, or increased or more efficient production.

The term "heterologous biosynthetic pathway" as used herein refers to an enzyme pathway to produce a product in which at least one of the enzymes is not endogenous to the host cell containing the biosynthetic pathway.

The term "extractant" as used herein refers to one or more organic solvents which can be used to extract butanol from a fermentation broth.

The term "effective isobutanol productivity" as used herein refers to the total amount in grams of isobutanol produced per gram of cells.

The term "effective titer" as used herein, refers to the total amount of a particular alcohol (e.g. butanol) produced by fermentation per liter of fermentation medium. The total amount of butanol includes: (i) the amount of butanol in the fermentation medium; (ii) the amount of butanol recovered from the organic extractant; and (iii) the amount of butanol recovered from the gas phase, if gas stripping is used.

The term "effective rate" as used herein, refers to the total amount of butanol produced by fermentation per liter of fermentation medium per hour of fermentation.

The term "effective yield" as used herein, refers to the amount of butanol produced per unit of fermentable carbon substrate consumed by the biocatalyst.

The term "separation" as used herein is synonymous with "recovery" and refers to removing a chemical compound from an initial mixture to obtain the compound in greater purity or at a higher concentration than the purity or concentration of the compound in the initial mixture.

The term "aqueous phase," as used herein, refers to the aqueous phase of a biphasic mixture obtained by contacting a fermentation broth with a water-immiscible organic extractant. In an embodiment of a process described herein that includes fermentative extraction, the term "fermentation broth" then specifically refers to the aqueous phase in biphasic fermentative extraction.

The term "organic phase," as used herein, refers to the non-aqueous phase of a biphasic mixture obtained by contacting a fermentation broth with a water-immiscible organic extractant.

The terms "PDC-," "PDC knockout," or "PDC-KO" as used herein refer to a cell that has a genetic modification to inactivate or reduce expression of a gene encoding pyruvate decarboxylase (PDC) so that the cell substantially or completely lacks pyruvate decarboxylase enzyme activity. If the cell has more than one expressed (active) PDC gene, then each of the active PDC genes can be inactivated or have minimal expression thereby producing a PDC-cell.

The term "polynucleotide" is intended to encompass a singular nucleic acid as well as plural nucleic acids, and refers to a nucleic acid molecule or construct, e.g., messenger RNA (mRNA) or plasmid DNA (pDNA). A polynucleotide can contain the nucleotide sequence of the full-length cDNA sequence, or a fragment thereof, including the untranslated 5° and 3' sequences and the coding sequences. The polynucleotide can be composed of any polyribonucleotide or polydeoxyribonucleotide, which can be unmodified RNA or DNA or modified RNA or DNA. For example, polynucleotides can be composed of single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that can be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. "Polynucleotide" embraces chemically, enzymatically, or metabolically modified forms.

A polynucleotide sequence can be referred to as "isolated," in which it has been removed from its native environment. For example, a heterologous polynucleotide encoding a polypeptide or polypeptide fragment having dihydroxy-acid dehydratase activity contained in a vector is considered isolated for the purposes of the present invention. Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) polynucleotides in solution. Isolated polynucleotides or nucleic acids according to the present invention further include such molecules produced synthetically. An isolated polynucleotide fragment in the form of a polymer of DNA can be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

The term "NAD(P)H consumption assay" refers to an enzyme assay for the determination of the specific activity of the KARI enzyme, involving measuring the disappearance of the KARI cofactor, NAD(P)H, from the enzyme reaction. Such assays are described in Aulabaugh and Schloss, Biochemistry 29: 2824-2830, 1990, which is herein incorporated by reference in its entirety.

The term "NAD(P)H" refers to either NADH or NADPH.

"KARI" is the abbreviation for the enzyme ketol-acid reductoisomerase.

The term "close proximity" when referring to the position of various amino acid residues of a KARI enzyme with respect to the adenosyl 2'-phosphate of NADPH means amino acids in the three-dimensional model for the structure of the enzyme that are within about 4.5 Å of the phosphorus atom of the adenosyl 2'-phosphate of NADPH bound to the enzyme.

The term "ketol-acid reductoisomerase" (abbreviated "KARI"), and "acetohydroxy acid isomeroreductase" will be used interchangeably and refer to enzymes capable of catalyzing the reaction of (S)-acetolactate to 2,3-dihydroxyisovalerate, classified as EC number EC 1.1.1.86 (Enzyme Nomenclature 1992, Academic Press, San Diego). As used herein the term "Class I ketol-acid reductoisomerase enzyme" means the short form that typically has between 330 and 340 amino acid residues, and is distinct from the long form, called class II, that typically has approximately 490 residues. These enzymes are available from a number of sources, including, but not limited to *E. coli* (amino acid SEQ ID NO: 942; GenBank Accession Number NC_000913 REGION: 3955993.3957468), *Vibrio cholerae* (GenBank Accession Number NC_002505 REGION: 157441.158925), *Pseudomonas aeruginosa*, (GenBank Accession Number NC_002516, REGION: 5272455.5273471), and *Pseudomonas fluorescens* (amino acid SEQ ID NO: 943; GenBank Accession Number NC_004129 REGION: 6017379.6018395). KARI enzymes are described for example, in U.S. Pat. Nos. 7,910,342 and 8,129,162 and U.S. Pub. App. No. 2010/0197519, all of which are herein incorporated by reference in their entireties.

KARI is found in a variety of organisms and amino acid sequence comparisons across species have revealed that there are 2 types of this enzyme: a short form (class I) found in fungi and most bacteria, and a long form (class II) typical of plants, Class I KARIs typically have between 330-340 amino acid residues. The long form KARI enzymes have about 490 amino acid residues. However, some bacteria such as *Escherichia* coil possess a long form, where the amino acid sequence differs appreciably from that found in plants. KARI is encoded by the ilvC gene and is an essential enzyme for growth of *E. coli* and other bacteria in a minimal medium. Class II KARIs generally consist of a 225-residue N-terminal domain and a 287-residue C-terminal domain. The N-terminal domain, which contains the NADPH-binding site, has an αβstructure and resembles domains found in other pyridine nucleotide-dependent oxidoreductases. The C-terminal domain consists almost entirely of α-helices.

Ketol-acid reductoisomerase (KARI) enzymes are useful in pathways for the production of isobutanol using engineered microorganisms (U.S. Pat. Nos. 7,851,188 and 7,993,889, incorporated by reference herein).

A KARI that can utilize NADH can capitalize on the NADH produced by the existing glycolytic and other meta-

bolic pathways in most commonly used microbial cells and can result in improved isobutanol production. Rane et al. (Arch. Biochem. Biophys., 338: 83-89, 1997) discusses cofactor switching of a ketol acid reductoisomerase isolated from *E. coli*. US Appl. Pub. Nos. 2009/0163376 and 2010/0197519 (each of which is herein incorporated by reference it its entirety) describe the generation of KARI enzymes which can use NADH. US Appl. Pub. No. 2010/0143997 (which is herein incorporated by reference in its entirety) describes *E. coli* variants with improved K_M values for NADH.

The terms "ketol-acid reductoisomerase activity" and "KARI activity" refer to the ability to catalyze the substrate to product conversion (S)-acetolactate to 2,3-dihydroxyisovalerate.

The term "acetolactate synthase" refers to an enzyme that catalyzes the conversion of pyruvate to acetolactate and CO_2. Acetolactate has two stereoisomers ((R) and (S)); the enzyme prefers the (S)-isomer, which is made by biological systems. Certain acetolactate synthases are known by the EC number 2.2.1.6 (Enzyme Nomenclature 1992, Academic Press, San Diego). These enzymes are available from a number of sources, including, but not limited to, *Bacillus subtilis* (GenBank Nos: CAB15618, Z99122, NCBI (National Center for Biotechnology Information) amino acid sequence, NCBI nucleotide sequence, respectively), *Klebsiella pneumoniae* (Gen Bank Nos: AAA25079, M73842 and *Lactococcus lactis* (GenBank Nos: AAA25161, L16975).

The term "acetohydroxy acid dehydratase" refers to an enzyme that catalyzes the conversion of 2,3-dihydroxyisovalerate to α-ketoisovalerate. Certain acetohydroxy acid dehydratases are known by the EC number 4.2.1.9. These enzymes are available from a vast array of microorganisms, including, but not limited to, *E. coli* (GenBank Nos: YP_026248, NC_000913, *S. cerevisiae* (GenBank Nos: NP_012550, NC_001142), *M. maripaludis* (GenBank Nos: CAF29874, BX957219), *B. subtilis* (GenBank Nos: CAB14105, Z99115), *Lactococcus lactis* (SEQ ID NO: 926), and *Streptococcus mutans* (SEQ ID NO: 939).

The term "branched-chain α-keto acid decarboxylase" refers to an enzyme that catalyzes the conversion of α-ketoisovalerate to isobutyraldehyde and CO_2. Certain branched-chain α-keto acid decarboxylases are known by the EC number 4.1.1.72 and are available from a number of sources, including, but not limited to, *Lactococcus lactis* (GenBank Nos: AAS49166, AY548760; CAG34226, AJ746364, *Salmonella typhimurium* (GenBank Nos: NP-461346, NC-003197), *Clostridium acetobutylicum* (GenBank Nos: NP-149189, NC-001988), *Macrococcus caseolyticus* (SEQ ID NO: 940), and *Listeria grayi* (SEQ ID NO: 941).

The term "branched-chain alcohol dehydrogenase" refers to an enzyme that catalyzes the conversion of isobutyraldehyde to isobutanol. Certain branched-chain alcohol dehydrogenases are known by the EC number 1.1.1.265, but can also be classified under other alcohol dehydrogenases (specifically, EC 1.1.1.1 or 1.1.1.2). These enzymes utilize NADH (reduced nicotinamide adenine dinucleotide) and/or NADPH as electron donor and are available from a number of sources, including, but not limited to, *S. cerevisiae* (GenBank Nos: NP_010656, NC_001136; NP_014051, NC_001145), *E. coli* (GenBank No: NP_417484), *C. acetobutylicum* (GenBank Nos: NP_349892, NC_003030), a indica (amino acid SEQ ID NO: 945), *A. xylosoxidans* (amino acid SEQ ID NO: 944).

The term "branched-chain keto acid dehydrogenase" refers to an enzyme that catalyzes the conversion of α-ketoisovalerate to isobutyryl-CoA (isobutyryl-cofactor A), using NAD^+ (nicotinamide adenine dinucleotide) as electron acceptor. Certain branched-chain keto acid dehydrogenases are known by the EC number 1.2.4.4. These branched-chain keto acid dehydrogenases comprise four subunits, and sequences from all subunits are available from a vast array of microorganisms, including, but not limited to, *B. subtilis* (GenBank Nos: CAB14336, Z99116; CAB14335, Z99116; CAB14334, Z99116; and CAB14337, Z99116) and *Pseudomonas putida* (GenBank Nos: AAA65614, M57613; AAA65615, M57613; AAA65617, M57613; and AAA65618, M57613).

As used herein, "aldehyde dehydrogenase activity" refers to any polypeptide having a biological function of an aldehyde dehydrogenase, including the examples provided herein. Such polypeptides include a polypeptide that catalyzes the oxidation (dehydrogenation) of aldehydes. Such polypeptides include a polypeptide that catalyzes the conversion of isobutyraldehyde to isobutyric acid. Such polypeptides also include a polypeptide that corresponds to Enzyme Commission Numbers EC 1.2.1.3, EC 1.2.1.4 or EC 1.2.1.5. Such polypeptides can be determined by methods well known in the art and disclosed herein.

As used herein, "aldehyde oxidase activity" refers to any polypeptide having a biological function of an aldehyde oxidase, including the examples provided herein. Such polypeptides include a polypeptide that catalyzes carboxylic acids from aldehydes. Such polypeptides include a polypeptide that catalyzes the conversion of isobutyraldehyde to isobutyric acid. Such polypeptides also include a polypeptide that corresponds to Enzyme Commission Number EC 1.2.3.1. Such polypeptides can be determined by methods well known in the art and disclosed herein.

As used herein, "pyruvate decarboxylase activity" refers to the activity of any polypeptide having a biological function of a pyruvate decarboxylase enzyme, including the examples provided herein. Such polypeptides include a polypeptide that catalyzes the conversion of pyruvate to acetaldehyde. Such polypeptides also include a polypeptide that corresponds to Enzyme Commission Number 4.1.1.1. Such polypeptides can be determined by methods well known in the art and disclosed herein. A polypeptide having pyruvate decarboxylate activity can be, by way of example, PDC1, PDC5, PDC6, or any combination thereof.

As used herein, "acetolactate reductase activity" refers to the activity of any polypeptide having the ability to catalyze the conversion of acetolactate to DHMB. Such polypeptides can be determined by methods well known in the art and disclosed herein.

As used herein, "DHMB" refers to 2,3-dihydroxy-2-methyl butyrate. DHMB includes "fast DHMB," which has the 2S, 3S configuration, and "slow DHMB," which has the 2S, 3R configurate. See Kaneko et al., Phytochemistry 39: 115-120 (1995), which is herein incorporated by reference in is entirety and refers to fast DHMB as angliceric acid and slow DHMB as tigliceric acid.

As used herein, "reduced activity" refers to any measurable decrease in a known biological activity of a polypeptide when compared to the same biological activity of the polypeptide prior to the change resulting in the reduced activity. Such a change can include a modification of a polypeptide or a polynucleotide encoding a polypeptide as described herein. A reduced activity of a polypeptide disclosed herein can be determined by methods well known in the art and disclosed herein.

As used herein, "eliminated activity" refers to the complete abolishment of a known biological activity of a polypeptide when compared to the same biological activity of the polypeptide prior to the change resulting in the eliminated activity. Such a change can include a modification of a polypeptide or a polynucleotide encoding a polypeptide as

described herein. An eliminated activity includes a biological activity of a polypeptide that is not measurable when compared to the same biological activity of the polypeptide prior to the change resulting in the eliminated activity. An eliminated activity of a polypeptide disclosed herein can be determined by methods well known in the art and disclosed herein.

The term "carbon substrate" or "fermentable carbon substrate" refers to a carbon source capable of being metabolized by host organisms of the present invention and particularly carbon sources selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and one-carbon substrates or mixtures thereof. Non-limiting examples of carbon substrates are provided herein and include, but are not limited to, monosaccharides, oligosaccharides, polysaccharides, ethanol, lactate, succinate, glycerol, carbon dioxide, methanol, glucose, fructose, sucrose, xylose, arabinose, dextrose, or mixtures thereof. Other carbon substrates can include ethanol, lactate, succinate, or glycerol.

"Fermentation broth" as used herein means the mixture of water, sugars (fermentable carbon sources), dissolved solids (if present), microorganisms producing alcohol, product alcohol and all other constituents of the material held in the fermentation vessel in which product alcohol is being made by the reaction of sugars to alcohol, water and carbon dioxide (CO_2) by the microorganisms present. From time to time, as used herein the term "fermentation medium" and "fermented mixture" can be used synonymously with "fermentation broth".

"Biomass" as used herein refers to a natural product containing a hydrolysable starch that provides a fermentable sugar, including any cellulosic or lignocellulosic material and materials comprising cellulose, and optionally further comprising hemicellulose, lignin, starch, oligosaccharides, disaccharides, and/or monosaccharides. Biomass can also comprise additional components, such as protein and/or lipids. Biomass can be derived from a single source, or biomass can comprise a mixture derived from more than one source. For example, biomass can comprise a mixture of corn cobs and corn stover, or a mixture of grass and leaves. Biomass includes, but is not limited to, bioenergy crops, agricultural residues, municipal solid waste, industrial solid waste, sludge from paper manufacture, yard waste, wood, and forestry waste. Examples of biomass include, but are not limited to, corn grain, corn cobs, crop residues such as corn husks, corn stover, grasses, wheat, rye, wheat straw, barley, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum, soy, components obtained from milling of grains, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, flowers, animal manure, and mixtures thereof.

"Feedstock" as used herein means a product containing a fermentable carbon source. Suitable feedstock include, but are not limited to, rye, wheat, corn, sugar cane, and mixtures thereof.

The term "aerobic conditions" as used herein means growth conditions in the presence of oxygen.

The term "microaerobic conditions" as used herein means growth conditions with low levels of oxygen (i.e., below normal atmospheric oxygen levels).

The term "anaerobic conditions" as used herein means growth conditions in the absence of oxygen.

The term "specific activity" as used herein is defined as the units of activity in a given amount of protein. Thus, the specific activity is not directly measured but is calculated by dividing 1) the activity in units/ml of the enzyme sample by 2) the concentration of protein in that sample, so the specific activity is expressed as units/mg, where an enzyme unit is defined as moles of product formed/minute. The specific activity of a sample of pure, fully active enzyme is a characteristic of that enzyme. The specific activity of a sample of a mixture of proteins is a measure of the relative fraction of protein in that sample that is composed of the active enzyme of interest.

The terms "k_{cat}" and "K_M" are known to those skilled in the art and are described in Enzyme Structure and Mechanism, 2^{nd} ed. (Ferst; W.H. Freeman Press, NY, 1985; pp 98-120), K_M, the Michaelis constant, is the concentration of substrate that leads to half-maximal velocity. The term "k_{cat}", often called the "turnover number", is defined as the maximum number of substrate molecules converted to products per active site per unit time, or the number of times the enzyme turns over per unit time. $k_{cat}=V_{max}/[E]$, where [E] is the enzyme concentration (Ferst, supra). The terms "total turnover" and "total turnover number" are used herein to refer to the amount of product formed by the reaction of a KARI enzyme with substrate.

The term "catalytic efficiency" is defined as the k_{cat}/K_M of an enzyme. Catalytic efficiency is used to quantify the specificity of an enzyme for a substrate.

The term "isolated nucleic acid molecule", "isolated nucleic acid fragment" and "genetic construct" will be used interchangeably and will mean a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of a polymer of DNA can be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

The term "amino acid" refers to the basic chemical structural unit of a protein or polypeptide. The following abbreviations are used herein to identify specific amino acids:

Amino Acid	Three-Letter Abbreviation	One-Letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

The term "gene" refers to a nucleic acid fragment that is capable of being expressed as a specific protein, optionally including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene can comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner

different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of a microorganism. A "foreign" gene refers to a gene not normally found in the host microorganism, but that is introduced into the host microorganism by gene transfer. Foreign genes can comprise native genes inserted into a non-native microorganism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

As used herein, "native" refers to the form of a polynucleotide, gene, or polypeptide as found in nature with its own regulatory sequences, if present.

As used herein the term "coding sequence" or "coding region" refers to a DNA sequence that encodes for a specific amino acid sequence. "Suitable regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences can include promoters, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing sites, effector binding sites and stem-loop structures.

As used herein, "endogenous" refers to the native form of a polynucleotide, gene or polypeptide in its natural location in the organism or in the genome of an organism. "Endogenous polynucleotide" includes a native polynucleotide in its natural location in the genome of an organism. "Endogenous gene" includes a native gene in its natural location in the genome of an organism. "Endogenous polypeptide" includes a native polypeptide in its natural location in the organism transcribed and translated from a native polynucleotide or gene in its natural location in the genome of an organism.

The term "heterologous" when used in reference to a polynucleotide, a gene, or a polypeptide refers to a polynucleotide, gene, or polypeptide not normally found in the host organism. "Heterologous" also includes a native coding region, or portion thereof, that is reintroduced into the source organism in a form that is different from the corresponding native gene, e.g., not in its natural location in the organism's genome. The heterologous polynucleotide or gene can be introduced into the host organism by, e.g., gene transfer. A heterologous gene can include a native coding region with non-native regulatory regions that is reintroduced into the native host. For example, a heterologous gene can include a native coding region that is a portion of a chimeric gene including non-native regulatory regions that is reintroduced into the native host. "Heterologous polypeptide" includes a native polypeptide that is reintroduced into the source organism in a form that is different from the corresponding native polypeptide.

A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

As used herein, the term "modification" refers to a change in a polynucleotide disclosed herein that results in reduced or eliminated activity of a polypeptide encoded by the polynucleotide, as well as a change in a polypeptide disclosed herein that results in reduced or eliminated activity of the polypeptide. Such changes can be made by methods well known in the art, including, but not limited to, deleting, mutating (e.g., spontaneous mutagenesis, random mutagenesis, mutagenesis caused by mutator genes, or transposon mutagenesis), substituting, inserting, down-regulating, altering the cellular location, altering the state of the polynucleotide or polypeptide (e.g., methylation, phosphorylation or ubiquitination), removing a cofactor, introduction of an antisense RNA/DNA, introduction of an interfering RNA/DNA, chemical modification, covalent modification, irradiation with UV or X-rays, homologous recombination, mitotic recombination, promoter replacement methods, and/or combinations thereof. Guidance in determining which nucleotides or amino acid residues can be modified, can be found by comparing the sequence of the particular polynucleotide or polypeptide with that of homologous polynucleotides or polypeptides, e.g., yeast or bacterial, and maximizing the number of modifications made in regions of high homology (conserved regions) or consensus sequences.

The term "recombinant genetic expression element" refers to a nucleic acid fragment that expresses one or more specific proteins, including regulatory sequences preceding (5' non-coding sequences) and following (3' termination sequences) coding sequences for the proteins. A chimeric gene is a recombinant genetic expression element. The coding regions of an operon can form a recombinant genetic expression element, along with an operably linked promoter and termination region.

"Regulatory sequences" refers to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences can include promoters, enhancers, operators, repressors, transcription termination signals, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing site, effector binding site and stem-loop structure.

The term "promoter" refers to a nucleic acid sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters can be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic nucleic acid segments. It is understood by those skilled in the art that different promoters can direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental or physiological conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". "Inducible promoters," on the other hand, cause a gene to be expressed when the promoter is induced or turned on by a promoter-specific signal or molecule. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths can have identical promoter activity. For example, it will be understood that "FBA1 promoter" can be used to refer to a fragment derived from the promoter region of the FBA1 gene.

The term "terminator" as used herein refers to DNA sequences located downstream of a coding sequence. This includes polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The 3' region can influence the transcription, RNA processing or stability, or translation of the associated coding sequence. It is recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths can have identical terminator activity. For example, it will be understood that "CYC1 terminator" can be used to refer to a fragment derived from the terminator region of the CYC1 gene.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of effecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the invention. Expression can also refer to translation of mRNA into a polypeptide.

The term "overexpression," as used herein, refers to expression that is higher than endogenous expression of the same or related gene. A heterologous gene is overexpressed if its expression is higher than that of a comparable endogenous gene. The term overexpression refers to an increase in the level of nucleic acid or protein in a host cell. Thus, overexpression can result from increasing the level of transcription or translation of an endogenous sequence in a host cell or can result from the introduction of a heterologous sequence into a host cell. Overexpression can also result from increasing the stability of a nucleic acid or protein sequence.

As used herein the term "transformation" refers to the transfer of a nucleic acid fragment into the genome of a host microorganism, resulting in genetically stable inheritance. Host microorganisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" microorganisms.

The terms "plasmid", "vector" and "cassette" refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA fragments. Such elements can be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. "Transformation cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitates transformation of a particular host cell. "Expression cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that allow for enhanced expression of that gene in a foreign host.

The term "site-saturation library" refers to a library which contains random substitutions at a specific amino acid position with up to and including all 20 possible amino acids at once.

The term "error-prone PCR" refers to adding random copying errors by imposing imperfect or 'sloppy' PCR reaction conditions which generate randomized libraries of mutations in a specific nucleotide sequence.

As used herein the term "codon degeneracy" refers to the nature in the genetic code permitting variation of the nucleotide sequence without affecting the amino acid sequence of an encoded polypeptide. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

The term "codon-optimized" as it refers to genes or coding regions of nucleic acid molecules for transformation of various hosts, refers to the alteration of codons in the gene or coding regions of the nucleic acid molecules to reflect the typical codon usage of the host organism without altering the polypeptide encoded by the DNA. Such optimization includes replacing at least one, or more than one, or a significant number, of codons with one or more codons that are more frequently used in the genes of that organism.

Deviations in the nucleotide sequence that comprise the codons encoding the amino acids of any polypeptide chain allow for variations in the sequence coding for the gene. Since each codon consists of three nucleotides, and the nucleotides comprising DNA are restricted to four specific bases, there are 64 possible combinations of nucleotides, 61 of which encode amino acids (the remaining three codons encode signals ending translation). The "genetic code" which shows which codons encode which amino acids is reproduced herein as Table 2A. As a result, many amino acids are designated by more than one codon. For example, the amino acids alanine and proline are coded for by four triplets, serine and arginine by six, whereas tryptophan and methionine are coded by just one triplet. This degeneracy allows for DNA base composition to vary over a wide range without altering the amino acid sequence of the proteins encoded by the DNA.

TABLE 2A

The Standard Genetic Code

	T	C	A	G
T	TTT Phe(F)	TCT Ser(S)	TAT Tyr(Y)	TGT Cys(C)
	TTC "	TCC "	TAC "	TGC
	TTA Leu(L)	TCA "	TAA Stop	TGA Stop
	TTG "	TCG "	TAG Stop	TGG Trp(W)
C	CTT Leu(L)	CCT Pro(P)	CAT His(H)	CGT Arg(R)
	CTC "	CCC "	CAC "	CGC "
	CTA "	CCA "	CAA Gln(Q)	CGA "
	CTG "	CCG "	GAG "	CGG "
A	ATT Ile(I)	ACT Thr(T)	AAT Asn(N)	AGT Ser(S)
	ATC "	ACC "	AAC "	AGC "
	ATA "	ACA "	AAA Lys(K)	AGA Arg(R)
	ATG Met(M)	ACG "	AAG "	AGG "
G	GTT Val(V)	GCT Ala(A)	GAT Asp(D)	GGT Gly(G)
	GTC "	GCC "	GAC "	GGC "
	GTA "	GCA "	GAA Glu(E)	GGA "
	GTG "	GCG "	GAG "	GGG "

Many organisms display a bias for use of particular codons to code for insertion of a particular amino acid in a growing peptide chain. Codon preference, or codon bias, differences in codon usage between organisms, is afforded by degeneracy of the genetic code, and is well documented among many organisms. Codon bias often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, inter alia, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization.

Given the large number of gene sequences available for a wide variety of animal, plant and microbial species, it is

possible to calculate the relative frequencies of codon usage. Codon usage tables are readily available, for example, at the "Codon Usage Database" available at www.kazusa.or.jp/codon/ (visited Mar. 20, 2008), and these tables can be adapted in a number of ways. See Nakamura, Y., et al. *Nucl. Acids Res.* 28:292 (2000). Codon usage tables for yeast, calculated from GenBank Release 128.0 [15 Feb. 2002], are reproduced below as Table 2B. This table uses mRNA nomenclature, and so instead of thymine (T) which is found in DNA, the tables use uracil (U) which is found in RNA. Table 2B has been adapted so that frequencies are calculated for each amino acid, rather than for all 64 codons.

TABLE 2B

Codon Usage Table for *Saccharomyces cerevisiae*

Amino Acid	Codon	Number	Frequency per thousand
Phe	UUU	170666	26.1
Phe	UUC	120510	18.4
Leu	UUA	170884	26.2
Leu	UUG	177573	27.2
Leu	CUU	80076	12.3
Leu	CUC	35545	5.4
Leu	CUA	87619	13.4
Leu	CUG	68494	10.5
Ile	AUU	196893	30.1
Ile	AUC	112176	17.2
Ile	AUA	116254	17.8
Met	AUG	136805	20.9
Val	GUU	144243	22.1
Val	GUC	76947	11.8
Val	GUA	76927	11.8
Val	GUG	70337	10.8
Ser	UCU	153557	23.5
Ser	UCC	92823	14.2
Ser	UCA	122028	18.7
Ser	UCG	55951	8.6
Ser	AGU	92466	14.2
Ser	AGC	63726	9.8
Pro	CCU	88263	13.5
Pro	CCC	44309	6.8
Pro	CCA	119641	18.3
Pro	CCG	34597	5.3
Thr	ACU	132522	20.3
Thr	ACC	83207	12.7
Thr	ACA	116084	17.8
Thr	ACG	52045	8.0
Ala	GCU	138358	21.2
Ala	GCC	82357	12.6
Ala	GCA	105910	16.2
Ala	GCG	40358	6.2
Tyr	UAU	122728	18.8
Tyr	UAC	96596	14.8
His	CAU	89007	13.6
His	CAC	50785	7.8
Gln	CAA	178251	27.3
Gln	CAG	79121	12.1
Asn	AAU	233124	35.7
Asn	AAC	162199	24.8
Lys	AAA	273618	41.9
Lys	AAG	201361	30.8
Asp	GAU	245641	37.6
Asp	GAC	132048	20.2
Glu	GAA	297944	45.6
Glu	GAG	125717	19.2
Cys	UGU	52903	8.1
Cys	UGC	31095	4.8
Trp	UGG	67789	10.4
Arg	CGU	41791	6.4
Arg	CGC	16993	2.6
Arg	CGA	19562	9.0
Arg	CGG	11351	1.7
Arg	AGA	139081	21.3
Arg	AGG	60289	9.2
Gly	GGU	156109	23.9
Gly	GGC	63903	9.8
Gly	GGA	71216	10.9
Gly	GGG	39359	6.0
Stop	UAA	6913	1.1
Stop	UAG	3312	0.5
Stop	UGA	4447	0.7

By utilizing this or similar tables, one of ordinary skill in the art can apply the frequencies to any given polypeptide sequence, and produce a nucleic acid fragment of a codon-optimized coding region which encodes the polypeptide, but which uses codons optimal for a given species.

Randomly assigning codons at an optimized frequency to encode a given polypeptide sequence, can be done manually by calculating codon frequencies for each amino acid, and then assigning the codons to the polypeptide sequence randomly. Additionally, various algorithms and computer software programs are readily available to those of ordinary skill in the art. For example, the "EditSeq" function in the Lasergene Package, available from DNAstar, Inc., Madison, Wis., the backtranslation function in the Vector NTI Suite, available from InforMax, Inc., Bethesda, Md., and the "backtranslate" function in the GCG-Wisconsin Package, available from Accelrys, Inc., San Diego, Calif. In addition, various resources are publicly available to codon-optimize coding region sequences, e.g., the "backtranslation" function (Entelechon GmbH, Regensburg, Germany) and the "backtranseq" function (NRC Saskatoon Bioinformatics, Saskatoon, Saskatchewan, Canada). Constructing a rudimentary algorithm to assign codons based on a given frequency can also easily be accomplished with basic mathematical functions by one of ordinary skill in the art.

Codon-optimized coding regions can be designed by various methods known to those skilled in the art including software packages such as "synthetic gene designer" (University of Maryland, Baltimore, Md.).

A polynucleotide or nucleic acid fragment is "hybridizable" to another nucleic acid fragment, such as a cDNA, genomic DNA, or RNA molecule, when a single-stranded form of the nucleic acid fragment can anneal to the other nucleic acid fragment under the appropriate conditions of temperature and solution ionic strength. Hybridization and washing conditions are well known and exemplified in Sambrook, J., Fritsch, E. F. and Maniatis, T. Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y. (1989), particularly Chapter 11 and

Table 11.1 therein (entirely incorporated herein by reference). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Stringency conditions can be adjusted to screen for moderately similar fragments (such as homologous sequences from distantly related organisms), to highly similar fragments (such as genes that duplicate functional enzymes from closely related organisms). Post hybridization washes determine stringency conditions. One set of conditions uses a series of washes starting with 6×SSC, 0.5% SDS at room temperature for 15 min, then repeated with 2×SSC, 0.5% SDS at 45° C. for 30 min, and then repeated twice with 0.2×SSC, 0.5% SDS at 50° C. for 30 min. Another set of stringent conditions uses higher temperatures in which the washes are identical to those above except for the temperature of the final two 30 min washes in 0.2× SSC, 0.5% SDS was increased to 60° C. Another set of highly stringent conditions uses two final washes in 0.1×SSC, 0.1% SDS at 65° C. An additional set of stringent conditions include hybridization at 0.1×SSC, 0.1% SDS, 65° C. and washes with 2×SSC, 0.1% SDS followed by 0.1×SSC, 0.1% SDS, for example.

Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of Tm for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher Tm) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating Tm have been derived (see Sambrook et al., supra, 9.50 9.51). For hybridizations with shorter nucleic acids, i.e., oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook et al., supra, 11.7 11.8). In one embodiment the length for a hybridizable nucleic acid is at least about 10 nucleotides. In one embodiment, a minimum length for a hybridizable nucleic acid is at least about 15 nucleotides; at least about 20 nucleotides; or the length is at least about 30 nucleotides. Furthermore, the skilled artisan will recognize that the temperature and wash solution salt concentration can be adjusted as necessary according to factors such as length of the probe.

As used herein, the term "polypeptide" is intended to encompass a singular "polypeptide" as well as plural "polypeptides," and refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). The term "polypeptide" refers to any chain or chains of two or more amino acids, and does not refer to a specific length of the product. Thus, peptides, dipeptides, tripeptides, oligopeptides, "protein," "amino acid chain," or any other term used to refer to a chain or chains of two or more amino adds, are included within the definition of "polypeptide," and the term "polypeptide" can be used instead of, or interchangeably with any of these terms. A polypeptide can be derived from a natural biological source or produced by recombinant technology, but is not necessarily translated from a designated nucleic acid sequence. It can be generated in any manner, including by chemical synthesis.

By an "isolated" polypeptide or a fragment, variant, or derivative thereof is intended a polypeptide that is not in its natural milieu. No particular level of purification is required. For example, an isolated polypeptide can be removed from its native or natural environment. Recombinantly produced polypeptides and proteins expressed in host cells are considered isolated for purposed of the invention, as are native or recombinant polypeptides which have been separated, fractionated, or partially or substantially purified by any suitable technique.

As used herein, the terms "variant" and "mutant" are synonymous and refer to a polypeptide differing from a specifically recited polypeptide by one or more amino acid insertions, deletions, mutations, and substitutions, created using, e.g., recombinant DNA techniques, such as mutagenesis. Guidance in determining which amino acid residues can be replaced, added, or deleted without abolishing activities of interest, can be found by comparing the sequence of the particular polypeptide with that of homologous polypeptides, e.g., yeast or bacterial, and minimizing the number of amino acid sequence changes made in regions of high homology (conserved regions) or by replacing amino acids with consensus sequences.

"Engineered polypeptide" as used herein refers to a polypeptide that is synthetic, i.e., differing in some manner from a polypeptide found in nature.

Alternatively, recombinant polynucleotide variants encoding these same or similar polypeptides can be synthesized or selected by making use of the "redundancy" in the genetic code. Various codon substitutions, such as silent changes which produce various restriction sites, can be introduced to optimize cloning into a plasmid or viral vector for expression. Mutations in the polynucleotide sequence can be reflected in the polypeptide or domains of other peptides added to the polypeptide to modify the properties of any part of the polypeptide. For example, mutations can be used to reduce or eliminate expression of a target protein and include, but are not limited to, deletion of the entire gene or a portion of the gene, inserting a DNA fragment into the gene (in either the promoter or coding region) so that the protein is not expressed or expressed at lower levels, introducing a mutation into the coding region which adds a stop codon or frame shift such that a functional protein is not expressed, and introducing one or more mutations into the coding region to alter amino acids so that a non-functional or a less enzymatically active protein is expressed.

Amino acid "substitutions" can be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, i.e., conservative amino acid replacements, or they can be the result of replacing one amino acid with an amino acid having different structural and/or chemical properties, i.e., non-conservative amino acid replacements. "Conservative" amino acid substitutions can be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino adds include aspartic acid and glutamic acid. Alternatively, "non-conservative" amino acid substitutions can be made by selecting the differences in polarity, charge, solubility, hydrophobicity, hydrophilicity, or the amphipathic nature of any of these amino acids. "Insertions" or "deletions" can be within the range of variation as structurally or functionally tolerated by the recombinant proteins. The variation allowed can be experimentally determined by systematically making insertions, deletions, or substitutions of amino acids in a polypeptide

molecule using recombinant DNA techniques and assaying the resulting recombinant variants for activity.

A "substantial portion" of an amino acid or nucleotide sequence is that portion comprising enough of the amino acid sequence of a polypeptide or the nucleotide sequence of a gene to putatively identify that polypeptide or gene, either by manual evaluation of the sequence by one skilled in the art, or by computer-automated sequence comparison and identification using algorithms such as BLAST (Altschul, S. F., et al., *J. Mol. Biol.,* 215:403-410 (1993)). In general, a sequence of ten or more contiguous amino acids or thirty or more nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene specific oligonucleotide probes comprising 20-30 contiguous nucleotides can be used in sequence-dependent methods of gene identification (e.g., Southern hybridization) and isolation (e.g., in situ hybridization of bacterial colonies or bacteriophage plaques). In addition, short oligonucleotides of 12-15 bases can be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of a nucleotide sequence comprises enough of the sequence to specifically identify and/or isolate a nucleic acid fragment comprising the sequence. The instant specification teaches the complete amino acid and nucleotide sequence encoding particular proteins. The skilled artisan, having the benefit of the sequences as reported herein, can now use all or a substantial portion of the disclosed sequences for purposes known to those skilled in this art. Accordingly, the instant invention comprises the complete sequences as reported in the accompanying Sequence Listing, as well as substantial portions of those sequences as defined above.

The term "complementary" is used to describe the relationship between nucleotide bases that are capable of hybridizing to one another. For example, with respect to DNA, adenine is complementary to thymine and cytosine is complementary to guanine, and with respect to RNA, adenine is complementary to uracil and cytosine is complementary to guanine.

The term "percent identity", as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in: 1.) *Computational Molecular Biology* (Lesk, A. M., Ed.) Oxford University: NY (1988); 2.) *Biocomputing: Informatics and Genome Projects* (Smith, D. W., Ed.) Academic: NY (1993); 3.) *Computer Analysis of Sequence Data, Part I* (Griffin, A. M., and Griffin, H. G., Eds.) Humania: NJ (1994); 4.) *Sequence Analysis in Molecular Biology* (von Heinje, G., Ed.) Academic (1987); and 5.) *Sequence Analysis Primer* (Gribskov, M. and Devereux, J., Eds.) Stockton: NY (1991).

Methods to determine identity are designed to give the best match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Sequence alignments and percent identity calculations can be performed using the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, Wis.). Multiple alignments of the sequences are performed using the "Clustal method of alignment" which encompasses several varieties of the algorithm including the "Clustal V method of alignment" corresponding to the alignment method labeled Clustal V (described by Higgins and Sharp, *CABIOS.* 5:151-153 (1989); Higgins, D. G. et al., *Comput. App. Biosci.,* 8:189-191 (1992)) and found in the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc.). For multiple alignments, the default values correspond to GAP PENALTY=10 and GAP LENGTH PENALTY=10. Default parameters for pairwise alignments and calculation of percent identity of protein sequences using the Clustal method are KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are KTUPLE=2, GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. After alignment of the sequences using the Clustal V program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program. Additionally the "Clustal W method of alignment" is available and corresponds to the alignment method labeled Clustal W (described by Higgins and Sharp, *CABIOS.* 5:151-153 (1989); Higgins, D. G. et al., *Comput. Appl. Biosci.* 8:189-191 (1992)) and found in the MegAlign™ v6.1 program of the LASERGENE bioinformatics computing suite (DNASTAR Inc.). Default parameters for multiple alignment (GAP PENALTY=10, GAP LENGTH PENALTY=0.2, Delay Divergen Segs (%)=30, DNA Transition Weight=0.5, Protein Weight Matrix=Gonnet Series, DNA Weight Matrix=IUB). After alignment of the sequences using the Clustal W program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program.

It is well understood by one skilled in the art that many levels of sequence identity are useful in identifying polypeptides, such as from other species, wherein such polypeptides have the same or similar function or activity, or in describing the corresponding polynucleotides. Useful examples of percent identities include, but are not limited to: 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, or any integer percentage from 55% to 100% can be useful in describing the present invention, such as 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%. Suitable polynucleotide fragments not only have the above homologies but typically comprise a polynucleotide having at least 50 nucleotides, at least 100 nucleotides, at least 150 nucleotides, at least 200 nucleotides, or at least 250 nucleotides. Further, suitable polynucleotide fragments having the above homologies encode a polypeptide having at least 50 amino acids, at least 100 amino acids, at least 150 amino acids, at least 200 amino acids, or at least 250 amino acids.

The term "sequence analysis software" refers to any computer algorithm or software program that is useful for the analysis of nucleotide or amino acid sequences. "Sequence analysis software" can be commercially available or independently developed. Typical sequence analysis software will include, but is not limited to: 1.) the GCG suite of programs (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, Wis.); 2.) BLASTP, BLASTN, BLASTX (Altschul et al., *J. Mol. Biol.,* 215:403-410 (1990)); 3.) DNASTAR (DNASTAR, Inc. Madison, Wis.); 4.) Sequencher (Gene Codes Corporation, Ann Arbor, Mich.); and 5.) the FASTA program incorporating the Smith-Waterman algorithm (W. R. Pearson, *Comput. Methods Genome Res.*, [Proc. Int. Symp.] (1994), Meeting Date 1992, 111-20. Editor(s): Suhai, Sandor, Plenum: New York, N.Y.). Within the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of

the program referenced, unless otherwise specified. As used herein "default values" will mean any set of values or parameters that originally load with the software when first initialized.

Standard recombinant DNA and molecular cloning techniques are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989) (hereinafter "Maniatis"); and by Silhavy, T. J., Bennan, M. L. and Enquis L. W., *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1984); and by Ausubel, F. M. et al., *Current Protocols in Molecular Biology*, published by Greene Publishing Assoc. and Wiley-Interscience (1987). Additional methods used here are in *Methods in Enzymology*, Volume 194, *Guide to Yeast Genetics and Molecular and Cell Biology* (Part A, 2004, Christine Guthrie and Gerald R. Fink (Eds.), Elsevier Academic Press, San Diego, Calif.). Other molecular tools and techniques are known in the art and include splicing by overlapping extension polymerase chain reaction (PCR) (Yu, et al. (2004) Fungal Genet. Biol. 41:973-981), positive selection for mutations at the URA3 locus of *Saccharomyces cerevisiae* (Boeke, J. D. et al. (1984) Gen. Genet. 197, 345-346; M A Romanos, et al. Nucleic Acids Res. 1991 Jan. 11; 19(1): 187), the cre-lox site-specific recombination system as well as mutant lox sites and FLP substrate mutations (Sauer, B. (1987) Mol Cell Biol 7: 2087-2096; Senecoff, et al. (1988) Journal of Molecular Biology, Volume 201, Issue 2, Pages 405-421; Albert, et al. (1995) The Plant Journal. Volume 7, Issue 4, pages 649-659), "seamless" gene deletion (Akada, et al, (2006) Yeast; 23(5): 399-405), and gap repair methodology (Ma et al., *Genetics* 58:201-216; 1981).

The genetic manipulations of a recombinant host cell disclosed herein can be performed using standard genetic techniques and screening and can be made in any host cell that is suitable to genetic manipulation (*Methods in Yeast Genetics*, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp. 201-202).

In embodiments, a recombinant host cell disclosed herein can be any yeast or fungi host useful for genetic modification and recombinant gene expression including those yeast mentioned elsewhere herein, such as in Table 7. In other embodiments, a recombinant host cell can be a member of the genera *Issatchenkia, Zygosaccharomyces, Schizosaccharomyces, Dekkera, Torulopsis, Brettanomyces, Torulaspora, Hanseniaspora, Kluveromyces, Yarrowia*, and some species of *Candida*.

Polypeptides with KARI Activity

In some embodiments, the recombinant host cells and methods provided herein address a need that arises in the microbial production of isobutanol where the KARI enzyme performs a vital role. In the isobutanol biosynthetic pathway shown in FIG. **1**, the substrate to product conversion of acetolactate to dihydroxyisovalerate (DHIV) is catalyzed by the KARI enzyme. Disclosed in US Appl. Publication No. US2011/0244536 and incorporated by reference, are polypeptides having ketol-acid reductoisomerase activity that are members of the SLSL Clade of KARIs. Polypeptides having KARI activity disclosed therein were found to be effective for isobutanol production. The SLSL Clade of KARIs include those KARI enzymes listed in Table 3.

TABLE 3

Effective KARIs

Description	SEQ ID NO: Nucleic acid	SEQ ID NO: Amino acid
Staphylococcus capitis SK14	66	67
Staphylococcus epidermidis M23864-W1	68	69
Staphylococcus hominis SK119	134	135
Staphylococcus aureus subsp. *aureus* TCH130	70	71
Staphylococcus warneri L37603	72	73
Staphylococcus epidermidis W23144	74	75
Staphylococcus saprophyticus subsp. *Saprophyticus* ATCC15305	76	77
Staphylococcus camosus subsp. *Camosus* TM300	78	79
Listeria monocytogenes EGD-e	80	81
Listeria grayi DSM 20601	82	83
Enterococcus casseliflavus EC30	84	85
Enterococcus gallinarum EG2	86	87
Macrococcus caseolyticus JCSC5402	88	89
Streptococcus vestibularis	90	91
Streptococcus mutans UA159	92	93
Streptococcus gordonii str, *cgakkus* sybstr. CH1	94	95
Streptococcus suis 89/1591	96	97
Streptococcus infantarius subsp. *infantarius* ATCC BAA-102	98	99
Lactococcus lactis subsp *cremoris* MG1363	100	101
Lactococcus lactis	102	103
Leuconostoc mesenteroides subsp *mesenteroides* ATCC8293	104	105
Lactobacillus buchneri ATCC 11577	106	107
Staphylococcus haemolyticus JCSC1435	108	109
Staphylococcus epidermidis ATCC12228	110	111
Streptococcus pneumoniae CGSP14	112	113
Streptococcus pneumoniae TIGR4	114	115
Streptococcus sanguinis SK36	116	117
Streptococcus salivarius SK126	118	119
Streptococcus thermophilus LMD-9	120	121
Streptococcus pneumoniae CCRI 1974M2	122	123
Lactococcus lactis subsp. *lactis* II1403	124	125
Leuconostoc mesenteroides subsp *cremoris* ATCC19254	126	127
Leuconostoc mesenteroides subsp *cremoris*	128	129
Lactobacillus brevis subsp. *gravesensis* ATCC27305	130	131
Lactococcus lactis subsp *lactis* NCDO2118	132	133

As described and demonstrated herein, Applicants have discovered additional KARI enzymes and variants of the additional KARIs that result in isobutanol production comparable to and/or exceeding that observed with the KARI from *Lactococcus lactis*. Such KARI enzymes and variants result in comparable or higher isobutanol titer and/or higher effective isobutanol productivity when compared to that observed with *Lactococcus lactis* KARI in the same conditions. Accordingly, in embodiments, polypeptides having KARI activity that function in an isobutanol production pathway have isobutanol titer and/or effective isobutanol productivity comparable to or better than that with the *Lactococcus lactis* KARI (SEQ ID NO: 380).

Such polypeptides having KARI activity may thus be suitable for isobutanol production. It will be appreciated that using a combination of structural and sequence information available in the art, polypeptides comprising KARI activity and less than 100% identity to the exemplified sequences can be constructed for use in isobutanol biosynthetic pathways. For example, crystal structures of the *E. coli* KARI enzyme at 2.6 Å resolution have been solved (Tyagi, et al., Protein Sci., 14: 3089-3100, 2005) as has the structure of the *P. aeruginosa* KARI (Ahn, et al., J. Mol. Biol., 328: 505-515, 2003) and the KARI enzyme from spinach (Biou V., et al. The EMBO Journal, 16: 3405-3415, 1997). Furthermore, described

herein is a Profile HMM (provided herein; Table Z) prepared using amino acid sequences of 25 KARI proteins with experimentally verified function as outlined in Table 1. The KARIs were from *Pseudomonas fluorescens* Pf-5, *Sulfolobus solfataricus* P2, *Pyrobaculum aerophilum* str. IM2, *Natronomonas pharaonis* DSM 2160, *Bacillus subtilis* subsp. *subtilis* str. 168, *Corynebacterium glutamicum* ATCC 13032, *Phaeospirilum molischianum, Ralstonia solanacearum* GMI1000, *Zymomonas mobilis* subsp. *mobilis* ZM4, *Alkalilimnicola ehrlichei* MLHE-, *Campylobacter lari* RM2100, *Marinobacter aquaeolei* VT8, *Psychrobacter arcticus* 273-4, *Hahella chejuensis* KCTC 2396, *Thiobacillus denitrificans* ATCC 25259, *Azotobacter vinelandii* AvOP, *Pseudomonas syringae* pv. *syringae* B728a, *Pseudomonas syringae* pv. tomato str. DC3000, *Pseudomonas putida* KT2440, *Pseudomonas entomophila* L48, *Pseudomonas mendocina* ymp, *Pseudomonas aeruginosa* PAO1, *Bacillus cereus* ATCC 10987, *Bacillus cereus* ATCC 10987, and *Spinacia oleracea*. Any protein that matches the Profile HMM with an E value of $<10^{-3}$ using hmmsearch program in the HMMER package is expected to be a functional KARI.

Production of isobutanol is believed to utilize the glycolysis pathway present in the host microorganism. During the production of two molecules of pyruvate from glucose during glycolysis, there is net production of two molecules of NADH from NAD+ by the glyceraldehyde-3-phosphate dehydrogenase reaction. During the further production of one molecule of isobutanol from two molecules of pyruvate, there is net consumption of one molecule of NAD(P)H, by the KARI reaction, and one molecule of NAD(P)H by the isobutanol dehydrogenase reaction. The interconversion of NADH with NADPH is generally slow and inefficient in yeast; thus, NADPH to be consumed is generated by metabolism (for example, by the pentose phosphate pathway) consuming substrate in the process. Meanwhile, the cell strives to maintain homeostasis in the NAD+/NADH ratio, leading to the excess NADH produced in isobutanol production being consumed in wasteful reduction of other metabolic intermediates; e.g., by the production of glycerol (Bakker, et al., 2001. Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 25:15-37). Thus, an imbalance between NADH produced and NADPH consumed by the isobutanol pathway can lead to a reduction in the molar yield of isobutanol produced from glucose in two ways: 1) unnecessary operation of metabolism to produce NADPH, and 2) wasteful reaction of metabolic intermediates to maintain NAD+/NADH homeostasis. Polypeptides having KARI activity that function well in an isobutanol pathway and have a low K_M for NADH can be used to improve the production of isobutanol.

Also disclosed herein are substitutions to the KARI enzyme sequences provided in Table 3 and in Table 10 to produce variants with varying ability to utilize NADH as a cofactor. Such variants provide alternatives that may be employed to optimize the efficiency of a biosynthetic pathway utilizing KARI, such as an isobutanol biosynthetic pathway, for particular production conditions. Demonstrated in the Examples is isobutanol production under conditions switched from aerobic to anaerobic for variants of the K9 KARI enzyme derived from *Anaerostipes caccae* with differing abilities to utilize NADH. Thus, equipped with this disclosure, one of skill in the art will be able to produce recombinant host cells comprising a SLSL Glade KARI enzyme, or a an *Enterococcus gallinarum, Streptococcus thermophilus Lactococcus lactis* subsp. *cremoris* MG1363, *Bifidobacterium angulatum, Bifidobacterium dentium*, or *Anaerostipes caccae, Lactococcus lactis* KARI enzyme or a variant or active fragment thereof suited for a range of production conditions.

In some embodiments, provided herein is a polypeptide having KARI activity and having at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% identity to a KARI enzyme of Table 3 or Table 10, or Examples 16, 17, 21 and having a K_M for NADH less than about 300 μM, 100 μM, 50 μM, 20 μM, 10 μM, or 5 μM. In some embodiments, provided herein is an engineered polypeptide having KARI activity and having at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% identity to a KARI enzyme of Table 3, Table 10 or Examples 16, 17, 21. In some embodiments, such polypeptides have a K_M for NADH less than that of the corresponding native enzyme. In some embodiments, the ratio of K_M for NADH to K_M for NAPDH is less than 0.1, in some embodiments less than 1, in some embodiments less than 2, in some embodiments, less than 4.

KARI enzymes and variants thereof that are particularly suitable for isobutanol production include, but are not limited to, variants of a ketol-acid reductoisomerase from *Anaerostipes caccae* DSM 14662 (SEQ ID NO: 643): "K9G9" (SEQ ID NO: 644) and "K9D3" (SEQ ID NO: 645) which have K_M for NADH lower than that of the native enzyme (SEQ ID NO: 643).

Host cells provided herein may comprise a polypeptide having ketol-acid reductoisomerase activity. In embodiments, such polypeptides have at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% identity to SEQ ID NO: 643, an active variant thereof; or a KARI derived from *Anaerostipes caccae* DSM 14662, or an active variant thereof. In embodiments, the polypeptides have at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% identity to SEQ ID NO: 645 or 644. In embodiments, the polypeptides comprise SEQ ID NO: 645 or 644.

In some embodiments, polypeptides having KARI activity comprise at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% identity to the amino acid sequence of SEQ ID NO: 419 [JB4P], 427 [SB2], and all those variants listed in Tables 25 and 26. Such variants provide alternatives for optimizing the efficiency of the isobutanol biosynthetic pathway for particular production conditions. Demonstrated in the Examples is isobutanol production under conditions.

Identification of Additional Polypeptides Having KARI Activity

Described in Example 1 is a biodiversity screen of KARL-encoding genes from various bacterial and fungal species which revealed suitable KARIs for isobutanol production. Equipped with this disclosure, one of skill in the art will be readily able to identify additional suitable polypeptides having KARI activity.

The sequences of other polynucleotides, genes and/or polypeptides can be identified in the literature and in bioinformatics databases well known to the skilled person using sequences disclosed herein and available in the art. For example, such sequences can be identified through BLAST searching of publicly available databases with polynucleotide or polypeptide sequences provided herein. In such a method, identities can be based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

Additionally, polynucleotide or polypeptide sequences disclosed herein can be used to identify other KARI homologs in nature. For example, each of the KARI encoding nucleic acid fragments disclosed herein can be used to isolate genes encoding homologous proteins. Isolation of homologous genes using sequence-dependent protocols is well known in the art. Examples of sequence-dependent protocols include, but are not limited to (1) methods of nucleic acid hybridization; (2) methods of DNA and RNA amplification, as exemplified by various uses of nucleic acid amplification technologies [e.g., polymerase chain reaction (PCR), Mullis et al., U.S. Pat. No. 4,683,202; ligase chain reaction (LCR), Tabor et al., Proc. Acad. Sci. USA 82:1074 (1985); or strand displacement amplification (SDA), Walker et al., Proc. Natl. Acad. Sci. U.S.A., 89:392 (1992)]; and (3) methods of library construction and screening by complementation.

It will be appreciated that one of ordinary skill in the art, equipped with this disclosure, can generate active fragments of polypeptides provided herein, for example, by truncating polypeptides provided herein based on sequence alignments at the N-terminus and confirming KARI activity. In embodiments, *Anaerostipes caccae* KARIs and variants thereof provided herein are truncated at the N-terminus. In one embodiment, up to and including the first five amino acids are truncated from a polypeptide provided herein. In embodiments, the polypeptide is SEQ ID NO: 27 or a variant thereof. In one embodiment, a polypeptide having KARI activity comprises SEQ ID NO: 635, 637 (encoded by polynucleotide sequences SEQ ID NO: 636 and 638, respectively), K9_Annabel_SH (SEQ ID NO:862, protein SEQ ID NO:863) and K9_Zeke_SH (SEQ ID NO: 860, protein SEQ ID NO: 861), or any variant listed in Table 40.

Lowering K_M for NADH

As shown in FIG. **2** and Examples, mutations in the positions corresponding to 50, 52 and 53, and optionally 47, of the *Pseudomonas* fluorescens KARI in the KARI enzyme from *Anaerostipes caccae* result in KARIs with lowered K_M for NADH as compared to wild-type, verifying that mutations in these positions produce NADH accepting variants of highly effective KARIs. Further mutations of *Anaerostipes caccae* KARI, revealed positions which further lower the K_M for NADH.

As demonstrated herein (see Examples), substitution of amino acids in the phosphate binding region, particularly in two or more positions corresponding to positions 47, 50, 52, and 53 of PF5 KARI (SEQ ID NO: 5) results in lowered K_M for NADH. Therefore, provided herein are polypeptides derived from an organism listed herein, for example, in Tables 3 and 10 having KARI activity and comprising substitutions at at least two of the four positions corresponding to positions 47, 50, 52, and 53 of PF5 KARI as compared to the native amino acid sequence. Provided herein are polypeptides having KARI activity and comprising substitutions in the phosphate binding region. Provided herein are polypeptides having KARI activity and comprising substitutions at positions corresponding to S56 and S58 of K9 KARI (SEQ ID NO: 27). In some embodiments the substitution at the position corresponding to S56 is A. In some embodiments, the substitution at the position corresponding to S58 is D or E. In some embodiments, the substitution at the position corresponding to S53 is Q, E, P, or A. In some embodiments, the substitution at the position corresponding to S56 is V or D. In some embodiments, the substitution at the position corresponding to S58 is D or Q. In embodiments, the polypeptides further comprise a substitution at one or more positions corresponding to 186, N87, N107, T131, or T191 of K9 KARI (SEQ ID NO: 27). In some embodiments, the polypeptides comprise a substitution at at least 2, at least 3, at least 4, or all of the indicated positions. In some embodiments, the substitution at the position corresponding to 186 is T or V. In some embodiments, the substitution at the position corresponding to N87 is P. In some embodiments, the substitution at the position corresponding to N107 is S. In some embodiments, the substitution at the position corresponding to T131 is C, L, A, M or V. In some embodiments, the substitution at the position corresponding to T191 is A, S, O, C, or G.

In embodiments, the polypeptides comprise fewer than 10, 15, or substitutions with respect to the wild-type sequence. In embodiments, the polypeptides match the Profile HMM based on experimentally verified KARIs and given in Table Z with an E value less than $<10^{-3}$. Sequences can be compared to the profile HMM given in Table Z using hmmsearch (HMMER software package available from Janelia Farm Research Campus, Ashburn, Va.).

Additional polypeptides having KARI activity and lowered K_M for NADH can be obtained using methods described and demonstrated herein. For example, a polypeptide having KARI activity can be employed in the construction of a site-saturation gene library as described herein. Kits for construction of such gene libraries are commercially available (for example, from USB Corporation, Cleveland, Ohio, #78480.) Site-directed mutagenesis can also be carried out using commercially available kits (for example, the QuickChange II XL site directed mutagenesis kit, Catalog #200524, Stratagene, La Jolla, Calif.). Primer design for target sites for mutagenesis is well-known in the art, and multiple sequence alignment to identify the target sites is likewise well-known.

Once variants have been generated, KARI activity with NADH or NADPH can be readily assessed using methods known in the art and/or disclosed herein. For example, KARI activity can be determined by measuring the disappearance of the NADPH or NADH from the reaction at 340 nm or by determination of the Michaelis constant via measurement of formation of 2,3-dihydroxyisovalerate using HPLC/MS. Likewise, isobutanol production from a strain comprising variants can be confirmed.

Cofactor Specificity

To determine cofactor specificity, V_{max}/K_M ratios can be calculated for each cofactor at saturating acetolactate; those variants with a higher ratio for NADH will react at a higher rate with NADH than NADPH under conditions of equal-molar concentrations of the two cofactors and saturating acetolactate. V_{max} and K_M values for NADH and NADPH can be determined using methods known in the art and/or provided herein (see Example 16). For example, to determine V_{max} and K_M values for NADH and NADPH, the partially purified proteins can be assayed at various concentrations of NADH and NADPH.

As demonstrated herein (see Examples 16 and 18 and FIG. **8**), substitution of additional amino acids in K9G9 results in variants having increased specificity for NADH. Thus, provided herein are polypeptides comprising substitution at one or more or all of the positions corresponding to K57, Y53, and E74 of K9 KARI (SEQ ID NO: 27). Also provided herein are polypeptides comprising substitutions at one or more or all of the positions corresponding to Y53, K57, E74, N87 and K90, In embodiments, the substitution at the position corresponding to Y53 is F. In embodiments, the substitution at the position corresponding to K57 is E. In embodiments, the substitution at the position corresponding to E74 is G. In embodiments, the substitution at the position corresponding to N87 is P. In embodiments, the substitution at the position corresponding to K90 is M or L. In embodiments, the variants comprise substitutions of at least one position corresponding

to S56 or S58 of SEQ ID NO: 27 and further comprise at least one, at least two, at least three, or more than three further substitution(s) corresponding to positions of SEQ ID NO: 27 identified herein.

In embodiments, the polypeptides comprise fewer than 2, 3, 4, 5, 10, 15, or 20 substitutions with respect to the wild-type sequence. In embodiments, the polypeptides match the Profile HMM based on experimentally verified KARIs and given in Table Z with an E value less than $<10^{-3}$.

As demonstrated in the Examples, variants of K9SB2 (SEQ ID NO: 427) were generated and screened for variants with reduced NADPH affinity, revealing additional positions for substitution. Thus, in embodiments, polypeptides further comprise substitutions at one or more positions corresponding to F53, G55, A56, W59, F67, I84, L85, Q91, M94, and P135 of SEQ ID NO: 427. In embodiments, the substitution at position G55 is D or C, the substitution at position Q91 is L, the substitution at position A56 is T or V, the substitution at P135 is S, the substitution at position F53 is L, the substitution at position M94 is I, the substitution at position F67 is L or I, the substitution at position W59 is C, the substitution at position 184 is L, and the substitution at position L85 is M.

KARI Structure

Structural information useful in the identification and modification of polypeptides having KARI activity is provided in art, such as in the references described here as well as in the Profile HMM provided herewith in Table Z and in US App. Pub. Nos. 20100197519 and 20090163376, incorporated herein by reference

It was reported that phosphate p2' oxygen atoms of NADPH form hydrogen bonds with side chains of Arg162, Ser165 and Ser167 of spinach KARI (Biou V., et al. The EMBO Journal, 16: 3405-3415, 1997). Studies by Ahn et al., (J. Mol. Biol., 328: 505-515, 2003) had identified three NADPH phosphate binding sites (Arg47, Ser50 and Thr52) for *Pseudomonas aeruginosa* (PAO-KARI) following comparing its structure with that of the spinach KARI. The structure of PF5-KARI with bound NADPH, acetolactate and magnesium ions was built based on the crystal structure of *P. aeruginosa* PAO1-KARI (PDB ID 1NP3, Ahn H. J. et al., J. Mol. Biol., 328: 505-515, 2003) which has 92% amino acid sequence homology to PF5 KARI, PAO1-KARI structure is a homo-dodecamer and each dodecamer consists of six homo-dimers with extensive dimer interface. The active site of KARI is located in this dimer interface. The biological assembly is formed by six homo-dimers positioned on the edges of a tetrahedron resulting in a highly symmetrical dodecamer of 23 point group symmetry.

The model of PF5-KARI dimer was built based on the coordinates of monomer A and monomer B of PAO1-KARI and sequence of PF5-KARI using DeepView/Swiss PDB viewer (Guex, N. and Peitsch, M. C., Electrophoresis, 18: 2714-2723, 1997). This model was then imported to program O (Jones, T. A. et al, Acta Crystallogr. A 47: 110-119, 1991) on a Silicon Graphics system for further modification.

The structure of PAO1-KARI has no NADPH, substrate or inhibitor or magnesium in the active site. Therefore, the spinach KARI structure (PDB ID 1yve, Biou V. et al., The EMBO Journal, 16: 3405-3415, 1997), which has magnesium ions, NADPH and inhibitor (N-Hydroxy-N-isopropyloxamate) in the acetolacate binding site, was used to model these molecules in the active site. The plant KARI has very little sequence homology to either PF5- or PAO1 KARI (<20% amino acid identity), however the structures in the active site region of these two KARI enzymes are very similar. To overlay the active site of these two KARI structures, commands LSQ_ext, LSQ_improve, LSQ_mol in the program O were used to line up the active site of monomer A of spinach KARI to the monomer A of PF5 KARI model. The coordinates of NADPH, two magnesium ions and the inhibitor bound in the active site of spinach KARI were extracted and incorporated to molecule A of PF5 KARI. A set of the coordinates of these molecules were generated for monomer B of PF5 KARI by applying the transformation operator from monomer A to monomer B calculated by the program.

Because there is no NADPH in the active site of PAO1 KARI crystal structure, the structures of the phosphate binding loop region in the NADPH binding site (residues 44-45 in PAO1 KARI, 157-170 in spinach KARI) are very different between the two. To model the NADPH bound form, the model of the PF5-KARI phosphate binding loop (44-55) was replaced by that of 1yve (157-170). Any discrepancy of side chains between these two was converted to those in the PF5-KARI sequence using the mutate_replace command in program O, and the conformations of the replaced side-chains were manually adjusted. The entire NADPH/Mg/inhibitor bound dimeric PF5-KARI model went through one round of energy minimization using program CNX (ACCELRYS San Diego Calif. Burnger, A. T. and Warren, G. L., Acta Crystallogr., D 54: 905-921, 1998) after which the inhibitor was replaced by the substrate, acetolactate (AL), in the model.

Isobutanol Production

Host cells provided herein can comprise a polypeptide having ketol-acid reductoisomerase activity. As described and demonstrated herein, Applicants have discovered additional KARI enzymes and variants of the additional KARIs that result in isobutanol production comparable to and/or exceeding that observed with the KARI from *Lactococcus lactis* (see Examples). Accordingly, in embodiments, polypeptides having KARI activity that function in an isobutanol production pathway have effective isobutanol productivity and/or produce isobutanol at a titer comparable to or better than that with the *Lactococcus lactis* KARI (SEQ ID NO: 380). Such polypeptides are thus considered to be useful for isobutanol production, particularly in cells comprising isobutanol production pathways described herein. In embodiments, polypeptides provided herein have effective isobutanol productivity and/or produce isobutanol at a titer greater than or about equal to that observed with the *Lactococcus lactis* KARI (SEQ ID NO: 380) under the same conditions. In embodiments, polypeptides provided herein have effective isobutanol productivity greater than about 3 grams per gram of cells, greater than about 4, greater than about 5, or greater than about 6 grams per gram of cells after about 48 hours wherein at least the last about 24 hours of the 48 hours are under anaerobic conditions.

Furthermore, Applicants have discovered that variants of the polypeptides having KARI activity described above, including those with K_M for NADH lower than that of the unsubstituted polypeptide, provide advantages for isobutanol production under anaerobic conditions. While not wishing to be bound by theory, it is believed that such variants provide improved isobutanol production due to more effective use of NADH as reducing equivalents. In embodiments, isobutanol production employing such a variant provides reduced glycerol accumulation. In embodiments, the molar ratio of isobutanol to glycerol is increased for a variant of a polypeptide having KARI activity described above with K_M for NADH lower than that of the unsubstituted polypeptide. In embodiments, the molar ratio of isobutanol to glycerol is greater than 1. In embodiments, the molar ratio of isobutanol to glycerol is greater than 2. In embodiments, the molar ratio is greater than 3. In embodiments, the molar ratio is greater than 4, greater than 5, greater than 6, greater than 7, greater than 8, greater

than 9, greater than 10, greater than 12, or greater than 14. In embodiments, the molar ratio is in the range of about 1 to 5, about 1 to 10, about 2 to 8, about 5 to 10, about 5 to 15 about 10 to 15 or about 12 to 15.

As demonstrated in the Examples herein, as the biochemical specificity for the NADH cofactor, as defined by (NADH V_{max}/K_M)/(NADPH V_{max}/K_M) increases, there is an observed increase in the isobutanol/glycerol ratio, suggesting that the altered cofactor specificity led to diminished NADPH utilization and by-product formation.

Modification of Aldehyde Dehydrogenase

In embodiments of the invention, a recombinant host cell can comprise reduced or eliminated aldehyde dehydrogenase activity and an isobutanol biosynthetic pathway wherein the host cell produces butanol. In other embodiments, the recombinant host cell can comprise an isobutanol or a 1-butanol biosynthetic pathway as described further herein. In other embodiments, the isobutanol biosynthetic pathway can comprise a polynucleotide encoding a polypeptide that catalyzes a substrate to product conversion selected from the group consisting of: (a) pyruvate to acetolactate; (b) acetolactate to 2,3-dihydroxyisovalerate; (c) 2,3-dihydroxyisovalerate to 2-ketoisovalerate; (d) 2-ketoisovalerate to isobutyraldehyde; and (e) isobutyraldehyde to isobutanol. In other embodiments, the isobutanol biosynthetic pathway can comprise polynucleotides encoding polypeptides having acetolactate synthase, keto acid reductoisomerase, dihydroxy acid dehydratase, ketoisovalerate decarboxylase, and alcohol dehydrogenase activity. In other embodiments, the recombinant cell comprises a 1-butanol biosynthetic pathway. In other embodiments, the 1-butanol biosynthetic pathway comprises a polynucleotide encoding a polypeptide that catalyzes a substrate to product conversion selected from the group consisting of: (a) acetyl-CoA to acetoacetyl-CoA; (b) acetoacetyl-CoA to 3-hydroxybutyryl-CoA; (c) 3-hydroxybutyryl-CoA to crotonyl-CoA; (d) crotonyl-CoA to butyryl-CoA; (e) butyryl-CoA to butyraldehyde; (f) butyraldehyde to 1-butanol. In other embodiments, the 1-butanol biosynthetic pathway can comprise polynucleotides encoding polypeptides having activity.

In embodiments of the invention, a recombinant host cell can comprise a modification or disruption of a polynucleotide or gene encoding a polypeptide having aldehyde dehydrogenase activity or a modification or disruption of a polypeptide having aldehyde dehydrogenase activity. Many methods for genetic modification and disruption of target genes to reduce or eliminate expression are known to one of ordinary skill in the art and can be used to create a recombinant host cell disclosed herein. In other embodiments, the recombinant host cell can comprise a deletion, mutation, and/or substitution in an endogenous polynucleotide or gene encoding a polypeptide having aldehyde dehydrogenase activity or in an endogenous polypeptide having aldehyde dehydrogenase activity. Such modifications, disruptions, deletions, mutations, and/or substitutions can result in aldehyde dehydrogenase activity that is reduced or eliminated. Modifications that can be used include, but are not limited to, deletion of the entire gene or a portion of the gene encoding an aldehyde dehydrogenase protein, inserting a DNA fragment into the encoding gene (in either the promoter or coding region) so that the protein is not expressed or expressed at lower levels, introducing a mutation into the coding region which adds a stop codon or frame shift such that a functional protein is not expressed, and introducing one or more mutations into the coding region to alter amino acids so that a non-functional or a less active protein is expressed. In other embodiments, expression of a target gene can be blocked by expression of an antisense RNA or an interfering RNA, and constructs can be introduced that result in cosuppression. In other embodiments, the synthesis or stability of the transcript can be lessened by mutation. In embodiments, the efficiency by which a protein is translated from mRNA can be modulated by mutation. All of these methods can be readily practiced by one skilled in the art making use of the known or identified sequences encoding target proteins.

In other embodiments, DNA sequences surrounding a target aldehyde dehydrogenase coding sequence are also useful in some modification procedures and are available, for example, for yeasts such as *Saccharomyces cerevisiae* in the complete genome sequence coordinated by Genome Project ID9518 of Genome Projects coordinated by NCBI (National Center for Biotechnology Information) with identifying GOPID #13838. An additional non-limiting example of yeast genomic sequences is that of *Candida albicans*, which is included in GPID #10771, #10701 and #16373. Other yeast genomic sequences can be readily found by one of skill in the art in publicly available databases.

In other embodiments, DNA sequences surrounding a target aldehyde dehydrogenase coding sequence can be useful for modification methods using homologous recombination. In a non-limiting example of this method, aldehyde dehydrogenase gene flanking sequences can be placed bounding a selectable marker gene to mediate homologous recombination whereby the marker gene replaces the aldehyde dehydrogenase gene. In another non-limiting example, partial aldehyde dehydrogenase gene sequences and aldehyde dehydrogenase gene flanking sequences bounding a selectable marker gene can be used to mediate homologous recombination whereby the marker gene replaces a portion of the target aldehyde dehydrogenase gene. In embodiments, the selectable marker can be bounded by site-specific recombination sites, so that following expression of the corresponding site-specific recombinase, the resistance gene is excised from the aldehyde dehydrogenase gene without reactivating the latter. In embodiments, the site-specific recombination leaves behind a recombination site which disrupts expression of the aldehyde dehydrogenase protein. In other embodiments, the homologous recombination vector can be constructed to also leave a deletion in the aldehyde dehydrogenase gene following excision of the selectable marker, as is well known to one skilled in the art.

In other embodiments, deletions can be made to an aldehyde dehydrogenase target gene using mitotic recombination as described by Wach et al. (Yeast, 10:1793-1808; 1994). Such a method can involve preparing a DNA fragment that contains a selectable marker between genomic regions that can be as short as 20 bp, and which bound a target DNA sequence. In other embodiments, this DNA fragment can be prepared by PCR amplification of the selectable marker gene using as primers oligonucleotides that hybridize to the ends of the marker gene and that include the genomic regions that can recombine with the yeast genome. In embodiments, the linear DNA fragment can be efficiently transformed into yeast and recombined into the genome resulting in gene replacement including with deletion of the target DNA sequence (((as disclosed, for example, in Methods in Enzymology, Volume 194, Guide to Yeast Genetics and Molecular and Cell Biology (Part A, 2004, Christine Guthrie and Gerald R. Fink (Eds.), Elsevier Academic Press, San Diego, Calif.)).

Moreover, promoter replacement methods can be used to exchange the endogenous transcriptional control elements allowing another means to modulate expression such as described by Mnaimneh et al., ((2004) Cell 118(1):31-44).

In other embodiments, the aldehyde dehydrogenase target gene encoded activity can be disrupted using random mutagenesis, which can then be followed by screening to identify strains with reduced or substantially eliminated activity. In this type of method, the DNA sequence of the target gene encoding region, or any other region of the genome affecting carbon substrate dependency for growth, need not be known. In embodiments, a screen for cells with reduced aldehyde dehydrogenase activity, or other mutants having reduced aldehyde dehydrogenase activity, can be useful for recombinant host cells of the invention.

Methods for creating genetic mutations are common and well known in the art and can be applied to the exercise of creating mutants. Commonly used random genetic modification methods (reviewed in Methods in Yeast Genetics, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) include spontaneous mutagenesis, mutagenesis caused by mutator genes, chemical mutagenesis, irradiation with UV or X-rays, or transposon mutagenesis.

Chemical mutagenesis of host cells can involve, but is not limited to, treatment with one of the following DNA mutagens: ethyl methanesulfonate (EMS), nitrous acid, diethyl sulfate, or N-methyl-N'-nitro-N-nitroso-guanidine (MNNG). Such methods of mutagenesis have been reviewed in Spencer et al. (Mutagenesis in Yeast, 1996, Yeast Protocols: Methods in Cell and Molecular Biology. Humana Press, Totowa, N.J.). In embodiments, chemical mutagenesis with EMS can be performed as disclosed in Methods in Yeast Genetics, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. Irradiation with ultraviolet (UV) light or X-rays can also be used to produce random mutagenesis in yeast cells. The primary effect of mutagenesis by UV irradiation is the formation of pyrimidine dimers which disrupt the fidelity of DNA replication. Protocols for UV-mutagenesis of yeast can be found in Spencer et al. (Mutagenesis in Yeast, 1996, Yeast Protocols: Methods in Cell and Molecular Biology. Humana Press, Totowa, N.J.). In embodiments, the introduction of a mutator phenotype can also be used to generate random chromosomal mutations in host cells. In embodiments, common mutator phenotypes can be obtained through disruption of one or more of the following genes: PMS1, MAG1, RAD18 or RAD51. In other embodiments, restoration of the non-mutator phenotype can be obtained by insertion of the wildtype allele. In other embodiments, collections of modified cells produced from any of these or other known random mutagenesis processes can be screened for reduced or eliminated aldehyde dehydrogenase activity.

Genomes have been completely sequenced and annotated and are publicly available for the following yeast strains: *Ashbya gossypii* ATCC 10895, *Candida glabrata* CBS 138, *Kiuyveromyces lactis* NRRL Y-1140, *Pichia stipitis* CBS 6054, *Saccharomyces cerevisiae* S288c, *Schizosaccharomyces pombe* 972h-, and *Yarrowia lipolytica* CLIB122. Typically BLAST (described above) searching of publicly available databases with known aldehyde dehydrogenase polynucleotide or polypeptide sequences, such as those provided herein, is used to identify aldehyde dehydrogenase-encoding sequences of other host cells, such as yeast cells.

In other embodiments, a polypeptide having aldehyde dehydrogenase activity can catalyze the conversion of isobutyraldehyde to isobutyric acid. In other embodiments, the conversion of isobutyraldehyde to isobutyric acid in a recombinant host cell is reduced or eliminated. In still other embodiments, a polynucleotide, gene or polypeptide having aldehyde dehydrogenase activity can correspond to Enzyme Commission Number EC 1.2.1.3, EC 1.2.1.4, and/or EC 1.2.1.5.

In embodiments, a recombinant host cell of the invention can be *S. carevisiae*, and a polypeptide having aldehyde dehydrogenase activity can be ALD2, ALD3, ALD4, ALD5, ALD6, or combinations thereof. In other embodiments, a recombinant host cell can be *Kluyveromyces lactis*, and a polypeptide having aldehyde dehydrogenase activity can be KLLA0F00440, KLLA0E23057, KLLA0D10021, KLLA0D09999G, or combinations thereof. In other embodiments, a recombinant host cell can be *Pichia stipitis*, and a polypeptide having aldehyde dehydrogenase activity can ALD2, ALD3, ALD4, ALD5, ALD7, or combinations thereof. In other embodiments, a recombinant host cell can be *Lactobacillus plantarum*, and a polypeptide having aldehyde dehydrogenase activity can be AldH. In other embodiments, a recombinant host cell can be *E. coli*, and a polypeptide having aldehyde dehydrogenase activity can be aldA, aldB, aldH, or combinations thereof.

In embodiments of the invention, a recombinant host cell can be *S. cerevisiae*, and an endogenous polynucleotide or gene encoding a polypeptide having aldehyde dehydrogenase activity can be ALD2, ALD3, ALD4, ALD5, ALD6, or combinations thereof. In embodiments of the invention, a recombinant host cell can be *S. cerevisiae*, and an endogenous polynucleotide or gene encoding a polypeptide having aldehyde dehydrogenase activity can be ALD6. In other embodiments, a recombinant host cell can be *Kiuyveromyces lactis*, and an endogenous polynucleotide or gene encoding a polypeptide having aldehyde dehydrogenase activity can be KLLA0F00440, KLLA0E23057, KLLA0D10021, KLLA0D09999G, or combinations thereof. In other embodiments, a recombinant host cell can be *Pichia stipitis*, and an endogenous polynucleotide or gene encoding a polypeptide having aldehyde dehydrogenase activity can be ALD2, ALD3, ALD4, ALD5, ALD7, or combinations thereof. In embodiments, the polypeptide having aldehyde dehydrogenase activity is a homolog of ALD6 from *Saccharomyces cerevisiae*. *S. cerevisiae* deletion strains containing aldehyde dehydrogenase gene deletions with a kanMX cassette are commercially available from American Type Culture Collection [catalog #4000753].

In other embodiments, a recombinant host cell can be *Lactobacillus plantarum*, and an endogenous polynucleotide encoding a polypeptide having aldehyde dehydrogenase activity can be AldH. In other embodiments, a recombinant host cell can be *E. coli*, and an endogenous polynucleotide encoding a polypeptide having aldehyde dehydrogenase activity can be aldA, aldB, aldH, or combinations thereof.

Examples of aldehyde dehydrogenase polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to, those of the following Table 4.

TABLE 4

Aldehyde dehydrogenase target gene coding regions and proteins.

	Nucleic acid SEQ ID NO:	Amino acid SEQ ID NO:
ALD2 from *S. cerevisiae*	732	733
ALD3 from *S. cerevisiae*	734	735
ALD4 from *S. cerevisiae*	736	737
ALD5 from *S. cerevisiae*	738	739
ALD6 from *S. cerevisiae*	740	741
KLLA0F00440 from *Kluyveromyces lactis*	742	743
KLLA0E23057 from *Kluyveromyces lactis*	744	745

TABLE 4-continued

Aldehyde dehydrogenase target gene coding regions and proteins.

	Nucleic acid SEQ ID NO:	Amino acid SEQ ID NO:
KLLA0D10021 from *Kluyveromyces lactis*	746	747
KLLA0D09999 from *Kluyveromyces lactis*	748	749
ALD2 from *Pichia stipits*	750	751
ALD3 from *Pichia stipitis*	752	753
ALD4 from *Pichia stipitis*	754	755
ALD5 from *Pichia stipitis*	756	757
ALD7 from *Pichia stipitis*	758	759
aldA from *E. coli*	760	761
aldB from *E. coli*	762	763
aldH (puuC) from *E. coli*	764	765

Other examples of aldehyde dehydrogenase polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to, aldehyde dehydrogenase polynucleotides, genes and/or polypeptides having at least about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 96%, about 97%, about 98%, or about 99% sequence identity to any one of the sequences of Table 4, wherein such a polynucleotide or gene encodes, or such a polypeptide has, aldehyde dehydrogenase activity. Still other examples of aldehyde dehydrogenase polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to an active variant, fragment or derivative of any one of the sequences of Table 4, wherein such a polynucleotide or gene encodes, or such a polypeptide has, aldehyde dehydrogenase activity.

In embodiments, the sequences of other aldehyde dehydrogenase polynucleotides, genes and/or polypeptides can be identified in the literature and in bioinformatics databases well known to the skilled person using sequences disclosed herein and available in the art. For example, such sequences can be identified through BLAST searching of publicly available databases with known aldehyde dehydrogenase-encoding polynucleotide or polypeptide sequences. In such a method, identities can be based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

Additionally, the aldehyde dehydrogenase polynucleotide or polypeptide sequences disclosed herein or known the art can be used to identify other aldehyde dehydrogenase homologs in nature. For example, each of the aldehyde dehydrogenase encoding nucleic acid fragments disclosed herein can be used to isolate genes encoding homologous proteins. Isolation of homologous genes using sequence-dependent protocols is well known in the art. Examples of sequence-dependent protocols include, but are not limited to (1) methods of nucleic acid hybridization; (2) methods of DNA and RNA amplification, as exemplified by various uses of nucleic acid amplification technologies [e.g., polymerase chain reaction (PCR). Mullis et al., U.S. Pat. No. 4,683,202; ligase chain reaction (LCR), Tabor et al., *Proc. Acad. Sci. USA* 82:1074 (1985); or strand displacement amplification (SDA), Walker et al., *Proc. Natl. Acad., Sci. U.S.A.,* 89:392 (1992)]; and (3) methods of library construction and screening by complementation.

Accordingly, it is within the scope of the invention to provide aldehyde dehydrogenase polynucleotides, genes and polypeptides having at least about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 96%, about 97%, about 98%, or about 99% sequence identity to any of the aldehyde dehydrogenase polynucleotides or polypeptides disclosed herein (e.g., SEQ ID NOs: 732-765 of Table 4) Identities are based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

The modification of aldehyde dehydrogenase in a recombinant host cell disclosed herein to reduce or eliminate aldehyde dehydrogenase activity can be confirmed using methods known in the art. For example, disruption of a particular aldehyde dehydrogenase could be confirmed with PCR screening using primers internal and external to the aldehyde dehydrogenase gene or by Southern blot using a probe designed to the aldehyde dehydrogenase gene sequence. Alternatively, one could utilize gas chromatography-mass spectroscopy or liquid chromatography to screen strains exposed to isobutyraldehyde for decreased formation of isobutyric acid. Accordingly, provided herein is a method of screening for strains with decreased isobutyric acid formation comprising: a) providing a strain comprising a modification in a polynucleotide encoding a polypeptide having aldehyde dehydrogenase activity and/or a modification in a polynucleotide encoding a polypeptide having aldehyde oxidase activity; b) contacting the cell with isobutyraldehyde; and c) measuring isobutyric acid formation; wherein isobutyric acid formation is reduced as compared to a control strain without the modification. In some embodiments, the modification is a deletion, mutation, and/or substitution. In some embodiments, the measuring is carried out using gas chromatography-mass spectroscopy. In some embodiments, isobutyric acid is reduced by at least about 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90%. In some embodiments, isobutyric acid formation is substantially eliminated.

Modification of Aldehyde Oxidase

In embodiments of the invention, a recombinant host cell disclosed herein can have a modification or disruption of a polynucleotide, gene or polypeptide encoding aldehyde oxidase. In embodiments, the recombinant host cell comprises a deletion, mutation, and/or substitution in an endogenous polynucleotide or gene encoding a polypeptide having aldehyde oxidase activity, or in an endogenous polypeptide having aldehyde oxidase activity. Such modifications, disruptions, deletions, mutations, and/or substitutions can result in aldehyde oxidase activity that is reduced or eliminated.

In embodiments of the invention, a polypeptide having aldehyde oxidase activity can catalyze the conversion of isobutyraldehyde to isobutyric acid. In other embodiments, the conversion of isobutyraldehyde to isobutyric acid in a recombinant host cell is reduced or eliminated. In other embodiments, a polynucleotide, gene or polypeptide having aldehyde oxidase activity can correspond to Enzyme Commission Number EC 1.2.3.1.

In embodiments, a recombinant host cell of the invention can be *Pichia stipitis* and a polynucleotide, gene or polypeptide having aldehyde oxidase activity can be AOX1 and/or AOX2.

Examples of aldehyde oxidase polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to, those of the following Table 5.

TABLE 5

Aldehyde oxidase target gene coding regions and proteins.

	Nucleic acid SEQ ID NO:	Amino acid SEQ ID NO:
AOX1 from *Pichia stipitis*	864	866
AOX2 from *Pichia stipitis*	867	868

Other examples of aldehyde oxidase polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to, aldehyde oxidase polynucleotides, genes and/or polypeptides having at least about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 96%, about 97%, about 98%, or about 99% sequence identity to any one of the sequences of Table 5, wherein such a polynucleotide or gene encodes a polypeptide having, or such a polypeptide has, aldehyde oxidase activity. Still other examples of aldehyde oxidase polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to an active variant, fragment or derivative of any one of the sequences of Table 5, wherein such a polynucleotide or gene encodes, or such a polypeptide has, aldehyde oxidase activity.

In embodiments, a polynucleotide, gene and/or polypeptide encoding an aldehyde oxidase sequence disclosed herein or known in the art can be modified, as disclosed above for acetolactate reductase or aldehyde dehydrogenase. In other embodiments, a polynucleotide, gene and/or polypeptide encoding aldehyde oxidase can be used to identify another aldehyde oxidase polynucleotide, gene and/or polypeptide sequence and/or can be used to identify an aldehyde oxidase homolog in other cells, as disclosed above for aldehyde dehydrogenase. Such aldehyde oxidase encoding sequences can be identified, for example, in the literature and/or in bioinformatics databases well known to the skilled person. For example, the identification of an aldehyde oxidase encoding sequence in another cell type using bioinformatics can be accomplished through BLAST (as disclosed above) searching of publicly available databases with a known hexose kinase encoding DNA and polypeptide sequence, such as any of those provided herein. Identities are based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

The modification of aldehyde oxidase in a recombinant host cell disclosed herein to reduce or eliminate aldehyde oxidase activity can be confirmed using methods known in the art. For example, disruption of a particular aldehyde oxidase could be confirmed with PCR screening using primers internal and external to the aldehyde oxidase gene or by Southern blot using a probe designed to the aldehyde oxidase gene sequence. Alternatively, one could utilize gas chromatography or other analytical methods to screen strains exposed to isobutyraldehyde for decreased formation of isobutyric acid (as described and demonstrated in the Examples). In some embodiments, isobutyric acid is reduced by at least about 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90%. In some embodiments, isobutyric acid formation is substantially eliminated.

Applicants have provided recombinant host cells comprising reduced or eliminated aldehyde dehydrogenase and/or aldehyde oxidase activity. In embodiments, a recombinant host cell disclosed herein can further comprise a modification in a polynucleotide encoding a polypeptide having pyruvate decarboxylase activity and/or a modification in a polynucleotide encoding a polypeptide having hexokinase 2 activity. In embodiments, a recombinant host cell of the invention can produce a production of a biosynthetic pathway (e.g., isobutanol), and can comprise a polynucleotide encoding a polypeptide that catalyzes a substrate to product conversion selected from the group consisting of: (a) pyruvate to acetolactate; (b) acetolactate to 2,3-dihydroxyisovalerate; (c) 2,3-dihydroxyisovalerate to 2-ketoisovalerate; (d) 2-ketoisovalerate to isobutyraldehyde; and (e) isobutyraldehyde to isobutanol. In other embodiments, such a recombinant host cell can produce a product of a biosynthetic pathway (e.g., isobutanol) at a yield or amount that is greater than the yield or amount of the same product produced by a recombinant host cell that does not comprise reduced or eliminated aldehyde dehydrogenase activity and/or aldehyde oxidase activity. In other embodiments, a recombinant host cell of the invention can reduce or eliminate the conversion of isobutyraldehyde to isobutyric acid, and can be used for screening candidate polypeptides having aldehyde dehydrogenase and/or aldehyde oxidase activity. As such, Applicants have also provided methods of increasing the yield or titer of a product of a biosynthetic pathway (e.g., isobutanol), methods for reducing or eliminating the conversion of isobutyraldehyde to isobutyric acid, and methods for screening candidate polypeptides having aldehyde dehydrogenase and/or aldehyde oxidase activity.

In embodiments of the invention, methods of producing a recombinant host cell are provided which comprise (a) providing a recombinant host cell disclosed herein; and (b) transforming said host cell with a polynucleotide encoding a polypeptide of a biosynthetic pathway (e.g., an isobutanol biosynthetic pathway). In other embodiments, methods of producing a recombinant host cell are provided which comprise (a) providing a recombinant host cell comprising a modification in a polynucleotide encoding a polypeptide having aldehyde dehydrogenase activity or in a polypeptide having aldehyde dehydrogenase activity; and (b) transforming said host cell with a polynucleotide encoding a polypeptide of a biosynthetic pathway (e.g., an isobutanol biosynthetic pathway). In other embodiments, methods of producing a recombinant host cell are provided which comprise (a) providing a recombinant host cell comprising a modification in a polynucleotide encoding a polypeptide having aldehyde oxidase activity or in a polypeptide having aldehyde oxidase activity; and (b) transforming said host cell with a polynucleotide encoding a polypeptide of an isobutanol biosynthetic pathway.

In embodiments, methods for reducing or eliminating the conversion of isobutyraldehyde to isobutyric acid are provided which comprise (a) providing a recombinant host cell disclosed herein; and (b) growing said host cell under conditions wherein the conversion of isobutyraldehyde to isobutyric acid is reduced or eliminated compared to a recombinant host cell that does not comprise reduced or eliminated aldehyde dehydrogenase and/or aldehyde oxidase activity. The conversion of isobutyraldehyde to isobutyric acid of a recombinant host cell disclosed herein can be measured by methods known in the art (see, e.g., Methods in Yeast Genetics, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp. 201-202) and/or described herein.

Reduction of DHMB

The production of DHMB in a host cell comprising an isobutanol biosynthetic pathway indicates that not all of the pathway substrates are being converted to the desired prod-

uct. Thus, yield is lowered. In addition, DHMB can have inhibitory effects on product production. For example, DHMB can decrease the activity of enzymes in the biosynthetic pathway or have other inhibitory effects on yeast growth and/or productivity during fermentation. Thus, the methods described herein provide ways of reducing DHMB during fermentation. The methods include both methods of decreasing the production of DHMB and methods of removing DHMB from fermenting compositions.

Decreasing DHMB Production

In some embodiments described herein, a recombinant host cell can comprise reduced or eliminated ability to convert acetolactate to DHMB. The ability of a host cell to convert acetolactate to DHMB can be reduced or eliminated, for example, by a modification or disruption of a polynucleotide or gene encoding a polypeptide having acetolactate reductase activity or a modification or disruption of a polypeptide having acetolactate reductase activity. In other embodiments, the recombinant host cell can comprise a deletion, mutation, and/or substitution in an endogenous polynucleotide or gene encoding a polypeptide having acetolactate reductase activity or in an endogenous polypeptide having acetolactate reductase. Such modifications, disruptions, deletions, mutations, and/or substitutions can result in acetolactate reductase activity that is reduced, substantially eliminated, or eliminated. In some embodiments of the invention, the product of the biosynthetic pathway is produced at a greater yield or amount compared to the production of the same product in a recombinant host cell that does not comprise reduced or eliminated ability to convert acetolactate to DHMB.

Thus, the product can be a composition comprising butanol that is substantially free of, or free of DHMB. In some embodiments, the composition comprising butanol contains no more than about 5 mM, about 4 mM, about 3 mM, about 2 mM, about 1 mM, about 0.5 mM, about 0.4 mM, about 0.3 mM DHMB, or about 0.2 mM DHMB.

The product can also be a composition comprising 2,3-butanediol (BDO) that is substantially free of, or free of DHMB. In some embodiments, the composition comprising BDO contains no more than about 5 mM, about 4 mM, about 3 mM, about 2 mM, about 1 mM, about 0.5 mM, about 0.4 mM, about 0.3 mM DHMB, or about 0.2 mM DHMB.

Any product of a biosynthetic pathway that involves the conversion of acetolactate to a substrate other than DHMB can be produced with greater effectiveness in a recombinant host cell disclosed herein having the described modification of acetolactate reductase activity. Such products include, but are not limited to, butanol, e.g., isobutanol, 2-butanol, and BDO, and branched chain amino acids.

In some embodiments, the host cell comprises at least one deletion, mutation, and/or substitution in at least one endogenous polynucleotide encoding a polypeptide having acetolactate reductase activity. In some embodiments, the host cell comprises at least one deletion, mutation, and/or substitution in each of at least two endogenous polynucleotides encoding polypeptides having acetolactate reductase activity.

In some embodiments, a polypeptide having acetolactate reductase activity can catalyze the conversion of acetolactate to DHMB. In some embodiments, a polypeptide having acetolactate reductase activity is capable of catalyzing the reduction of acetolactate to 2S,3S-DHMB (fast DHMB) and/or 2S,3R-DHMB (slow DHMB).

TABLE 6

Polypeptides and polynucleotides having acetolactate reductase activity in *Saccharomyces cerevisiae*

Gene	SEQ ID NO: (nucleic acid, amino acid)
YMR226C	676, 677
YIL074C (Chr 9)	678, 679
YIR036C (Chr 9)	680, 681
YPL061W (ALD6)(Chr 16)	682, 683
YPL088W(Chr 16)	684, 685
YCR105W (ADH7)(Chr 3)	686, 687
YDR541C(Chr 4)	688, 689
YER081 (SER3)(Chr 5)	690, 691
YPL275W (FDH2)(Chr 16)	692, 693
YBR006W (UGA5)(Chr2)	694, 695
YOL059W(Chr 15)	696, 697
YER081W (Chr 5)	869, 870
YOR375C (Chr 15)	871, 872

In some embodiments, the conversion of acetolactate to DHMB in a recombinant host cell is reduced, substantially eliminated, or eliminated. In some embodiments, the polypeptide having acetolactate reductase activity is selected from the group consisting of: YMR226C, YER081W, YIL074C, YBR006W, YPL275W, YOL059W, YIR036C, YPL061W, YPL088W, YCR105W, YOR375c, and YDR541C. In some embodiments, the polypeptide having acetolactate reductase activity is a polypeptide comprising a sequence listed in Table 6 or a sequence that is at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% identical to a polypeptide sequence listed in Table 6. In some embodiments, the polypeptide having acetolactate reductase activity is a polypeptide encoded by a polynucleotide sequence listed in Table 6 or a sequence that is at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% identical to a polynucleotide sequence listed in Table 6.

TABLE 7

Example YMR226C Yeast Homologs

Species	Accession #	SEQ ID NO: (nucleic acid, amino acid)
Saccharomyces paradoxus	AABY01000127	698, 699
Saccharomyces bayanus	AACA01000631	700, 701 MSQGRKAAERLANKTVLITGASA GIGKATALEYLEASNGNMKLILAA RRLEKLEELKKTIDEEFPNAKVH VGQLDITQAEKIKPFIENLPEAFK DIDILINNAGKALGSERVGDATQ DIQDVFDTNVTALINVTQAVLPIF QAKNSGDIVNLGLGGRQRRIPH RLHLLCFQVCRRCVH*QFEKGT

TABLE 7 -continued

Example YMR226C Yeast Homologs

Species	Accession #	SEQ ID NO: (nucleic acid, amino acid)
		DQHEDQSYLDRAGAG*DRVLTG QIQR**GTS*KRLQGHYAVDGRR RG*LNRIFHFQKAEHRGCRHPDL PHQPSLALPHLSRL* (SEQ ID NO: 701) The sequence came from a comparative genomics study using "draft" genome sequences with 7-fold coverage Kellis at al, Nature 423:241-254(2003)).
Saccharomyces castellii	AACF01000116	702, 703
Saccharomyces mikafae	AACH01000019	704, 705
Ashbya gossypii	AE016819	706, 707
Candida glabrata	CR380959	708, 709
Debaryomyces hansenii	CR382139	710, 711
Scheffersomyces stipitis (formerly *Pichia stipitis*)	XM_001387479	712, 713
Meyerozyma guilliermondii (formerly *Pichia guilliermondit*)	XM_0014 2184	714, 715
Vanderwaltozyma polyspora (formerly *Kluyveromyces polysporus*)	XM_001645671	716, 717
Candida dubliniensis	XM_002419771	718, 719
Zygosaccharomyces rouxii	XM_002494574	720, 721
Lachancea thermotolerans (formerly *Kluyveromyces thermotolerans*)	XM_002553230	722, 723
Kluyveromyces lactis	XM_451902	724, 725
Saccharomyces kluyveri	SAKL0H04730	726, 727
Yarrowia lipolytica	XM_501554	728, 729
Schizosaccharomyces pombe	NM_001018495	730, 731

In some embodiments, a polypeptide having acetolactate reductase activity is YMR226C or a homolog of YMR226C. Thus, in some embodiments, the polypeptide having acetolactate reductase activity is a polypeptide comprising a sequence listed in Table 7 or a sequence that is at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% identical to a polypeptide sequence listed in Table 7. In some embodiments, the polypeptide having acetolactate reductase activity is a polypeptide encoded by a polynucleotide sequence listed in Table 7 or a sequence that is at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% identical to a polynucleotide sequence listed in Table 7. Acetolactate reductases capable of converting acetolactate to DHMB can be identified, for example, by screening genetically altered yeast for changes in acetolactate consumption, changes in DHMB production, changes in DHIV production, or changes in other downstream product (e.g., butanol) production.

One way of identifying a gene involved in DHMB production comprises measuring the amount of DHMB produced by individual yeast strains in a yeast knock-out library. Knock-out libraries are available, for example, from Open Biosystems® (a division of Thermo Fisher Scientific, Waltham, Mass.). In this method, a decrease in DHMB production indicates that the gene that has been knocked-out functions to increase DHMB production, and an increase in DHMB production indicates that the gene that has been knocked-out functions to decrease DHMB production.

Two ways that a knockout ("KO") library can be used to identify candidate genes for involvement in DHMB synthesis include: (1) DHMB and DHIV accumulated in the culture during growth from endogenous substrates (acetolactate and NADPH or NADH) can be analyzed in samples from cultures. These samples can be placed in a hot (80-100° C.) water bath for 10-20 min, or diluted into a solution such as 2% formic acid that will kill and permeabilize the cells. After either treatment, small molecules will be found in the supernatant after centrifugation (5 min, 1100×g). The DHMB/

DHIV ratio of a control strain (e.g., BY4743) can be compared to that of the different KO derivatives, and the gene(s) missing from any strain(s) with exceptionally low DHMB/DHIV ratios can encode acetolactate reductase (ALR). (2) DHMB and/or DHIV formation rates in vitro from exogenous substrates (acetolactate and NADH and/or NADPH) can be measured in timed samples taken from a suspension of permeabilized cells, and inactivated in either of the ways described above. Since the substrates for DHMB and DHIV synthesis are the same, this allows one to measure the relative levels of ALR and KARI activity in the sample.

Another way of identifying a gene involved in DHMB production comprises measuring the amount of DHMB produced by individual yeast strains in a yeast overexpression library. Overexpression libraries are available, for example, from Open Biosystems® (a division of Thermo Fisher Scientific, Waltham, Mass.). In this method, a decrease in DHMB production indicates that the overexpressed gene functions to decrease DHMB production, and an increase in DHMB production indicates that the overexpressed gene functions to increase DHMB production.

Another way of identifying a gene involved in DHMB production is to biochemically analyze a DHMB-producing yeast strain. For example, DHMB-producing cells can be disrupted. This disruption can be performed at low pH and cold temperatures. The cell lysates can be separated into fractions, e.g., by adding ammonium sulfate or other techniques known to those of skill in the art, and the resulting fractions can be assayed for enzymatic activity. For example, the fractions can be assayed for the ability to convert acetolactate to DHMB. Fractions with enzymatic activity can be treated by methods known in the art to purify and concentrate the enzyme (e.g., dialysis and chromatographic separation). When a sufficient purity and concentration is achieved, the enzyme can be sequenced, and the corresponding gene encoding the acetolactate reductase capable of converting acetolactate to DHMB can be identified.

Furthermore, since the reduction of acetolactate to DHMB occurs in yeast, but does not occur to the same extent in *E. coli*, acetolactate reductases that are expressed in yeast, but not expressed in *E. coli*, can be selected for screening. Selected enzymes can be expressed in yeast or other protein expression systems and screened for the capability to convert acetolactate to DHMB.

Enzymes capable of catalyzing the conversion of acetolactate to DHMB can be screened by assaying for acetolactate levels, by assaying for DHMB levels, by assaying for DHIV levels, or by assaying for any of the downstream products in the conversion of DHIV to butanol, including isobutanol.

DHMB can be measured using any technique known to those of skill in the art. For example, DHMB can be separated and quantified by methods known to those of skill in the art and techniques described in the Examples provided herein. For example, DHMB can be separated and quantified using liquid chromatography-mass spectrometry, liquid chromatography-nuclear magnetic resonance (NMR), thin-layer chromatography, and/or HPLC with UV/Vis detection.

In embodiments, selected acetolactate reductase polynucleotides, genes and/or polypeptides disclosed herein can be modified or disrupted. Many suitable methods are known to those of ordinary skill in the art and include those described for aldehyde dehydrogenase (above).

The modification of acetolactate reductase in a recombinant host cell disclosed herein to reduce or eliminate acetolactate reductase activity can be confirmed using methods known in the art. For example, the presence or absence of an acetolactate reductase-encoding polynucleotide sequence can be determined using PCR screening. A decrease in acetolactate reductase activity can also be determined based on a reduction in conversion of acetolactate to DHMB. A decrease in acetolactate reductase activity can also be determined based on a reduction in DHMB production. A decrease in acetolactate reductase activity can also be determined based on an increase in butanol production.

Thus, in some embodiments, a yeast that is capable of producing butanol produces no more than about 5 mM, about 4 mM, about 3 mM, about 2 mM, about 1 mM, about 0.9 mM, about 0.8 mM., about 0.7 mM, about 0.6 mM, about 0.5 mM, about 0.4 mM or about 0.3 mM DHMB. In some embodiments, a yeast producing butanol produces no more than about 5 mM, about 4 mM, about 3 mM, about 2 mM, about 1 mM, about 0.9 mM, about 0.8 mM., about 0.7 mM, about 0.6 mM, about 0.5 mM, about 0.4 mM or about 0.3 mM DHMB. In some embodiments, a yeast producing butanol produces no more than about 0.2 mM or 0.2 mM DHMB.

In some embodiments, a yeast capable of producing butanol produces no more than about 10 mM DHMB when cultured under fermentation conditions for at least about 50 hours. In some embodiments, a yeast capable of producing butanol produces no more than about 5 mM DHMB when cultured under fermentation conditions for at least about 20 hours, at least about 25 hours, at least about 30 hours, at least about 35 hours, at least about 40 hours, at least about 45 hours, or at least about 50 hours. In some embodiments, a yeast capable of producing butanol produced no more than about 3 mM DHMB when cultured under fermentation conditions for at least about 5 hours, at least about 10 hours, at least about 15 hours, at least about 20 hours, at least about 25 hours, at least about 30 hours, at least about 35 hours, at least about 40 hours, at least about 45 hours, or at least about 50 hours. In some embodiments, a yeast capable of producing butanol produced no more than about 1 mM DHMB when cultured under fermentation conditions for at least about 1 hour, at least about 5 hours, at least about 10 hours, at least about 15 hours, at least about 20 hours, at least about 25 hours, at least about 30 hours, at least about 35 hours, at least about 40 hours, at least about 45 hours, or at least about 50 hours. In some embodiments, a yeast capable of producing butanol produced no more than about 0.5 mM DHMB when cultured under fermentation conditions for at least about 1 hour, at least about 5 hours, at least about 10 hours, at least about 15 hours, at least about 20 hours, at least about 25 hours, at least about 30 hours, at least about 35 hours, at least about 40 hours, at about 45 hours, or at least about 50 hours.

In some embodiments, a yeast comprising at least one deletion, mutation, and/or substitution in an endogenous polynucleotide encoding an acetolactate reductase produces no more than about 0.5 times, about 0.4 times, about 0.3 times, about 0.2 times, about 0.1 times, about 0.05 times the amount of DHMB produced by a yeast containing the endogenous polynucleotide encoding an acelotacatate reductase when cultured under fermentation conditions for the same amount of time. In some embodiments, a yeast that is capable of producing butanol produces an amount of DHIV that is at least about 5 mM, at least about 6 mM, at least about 7 mM, at least about 8 mM, at least about 9 mM, or at least about 10 mM.

In some embodiments, a yeast that is capable of producing butanol produces an amount of DHIV that is at least about the amount of DHMB produced. In some embodiments, a yeast that is capable of producing butanol produces an amount of DHIV that is at least about twice, about three times, about five times, about ten times, about 15 times, about 20 times, about 25 times, about 30 times, about 35 times, about 40 times,

about 45 times, or about 50 times the amount of DHMB produced. In some embodiments, a yeast that is capable of producing butanol produces DHIV at a rate that is at least about equal to the rate of DHMB production. In some embodiments, a yeast that is capable of producing butanol produces DHIV at a rate that is at least about twice, about three times, about five times, about ten times, about 15 times, about 20 times, about 25 times, about 30 times, about 35 times, about 40 times, about 45 times, or about 50 times the rate of DHMB production.

In some embodiments, a yeast that is capable of producing butanol produces less than 0.010 moles of DHMB per mole of glucose consumed. In some embodiments, a yeast produces less than about 0.009, less than about 0.008, less than about 0.007, less than about 0.006, or less than about 0.005 moles of DHMB per mole of glucose consumed. In some embodiments, a yeast produces less than about 0.004, less than about 0.003, less than about 0.002, or less than about 0.001 moles of DHMB per mole of glucose consumed.

In some embodiments, acetolactate reductase activity is inhibited by chemical means. For example, acetolactate reductase could be inhibited using other known substrates such as those listed in Fujisawa et al. including L-serine, D-serine, 2-methyl-DL-serine, D-threonine, L-allo-threonine, L-3-hydroxyisobutyrate, D-3-hydroxyisobutyrate, 3-hydroxypropionate, L-3-hydroxybutyrate, and D-3-hydroxybutyrate. Biochimica et Biophysica Acta 1645:89-94 (2003), which is herein incorporated by reference in its entirety.

DHMB Removal

In other embodiments described herein, a reduction in DHMB can be achieved by removing DHMB from a fermentation. Thus, fermentations with reduced DHMB concentrations are also described herein. Removal of DHMB can result, for example, in a product of greater purity, or a product requiring less processing to achieve a desired purity. Therefore, compositions comprising products of biosynthetic pathways such as ethanol or butanol with increased purity are also provided.

DHMB can be removed during or after a fermentation process and can be removed by any means known in the art. DHMB can be removed, for example, by extraction into an organic phase or reactive extraction.

In some embodiments, the fermentation broth comprises less than about 0.5 mM DHMB. In some embodiments, the fermentation broth comprises less than about 1.0 mM DHMB after about 5 hours, about 10 hours, about 15 hours, about 20 hours, about 25 hours, about 30 hours, about 35 hours, about 40 hours, about 45 hours, or about 50 hours of fermentation. In some embodiments, the fermentation broth comprises less than about 5.0 mM DHMB after about 20 hours, about 25 hours, about 30 hours, about 35 hours, about 40 hours, about 45 hours, or about 50 hours of fermentation.

Butanol Biosynthetic Pathways

Certain suitable isobutanol biosynthetic pathways are disclosed in U.S. Pat. Nos. 7,851,188 and 7,993,889, each of which is incorporated by reference herein. A diagram of the disclosed isobutanol biosynthetic pathways is provided in FIG. **1**. As described in U.S. Pat. No. 7,851,188, steps in an example isobutanol biosynthetic pathway include conversion of:

- pyruvate to acetolactate (see FIG. **1**, pathway step a therein), as catalyzed for example by acetolactate synthase (ALS),
- acetolactate to 2,3-dihydroxyisovalerate (see FIG. **1**, pathway step b therein) as catalyzed for example by acetohydroxy acid isomeroreductase (KARI);
- 2,3-dihydroxyisovalerate to 2-ketoisovalerate (see FIG. **1**, pathway step c therein) as catalyzed for example by acetohydroxy acid dehydratase, also called dihydroxyacid dehydratase (DHAD);
- 2-ketoisovalerate to isobutyraldehyde (see FIG. **1**, pathway step d therein) as catalyzed for example by branched-chain 2-keto acid decarboxylase; and
- isobutyraldehyde to isobutanol (see FIG. **1**, pathway step e therein) as catalyzed for example by branched-chain alcohol dehydrogenase.

In another embodiment, the isobutanol biosynthetic pathway comprises the following substrate to product conversions:

- pyruvate to acetolactate, which may be catalyzed, for example, by acetolactate synthase;
- acetolactate to 2,3-dihydroxyisovalerate, which may be catalyzed, for example, by ketol-acid reductoisomerase;
- 2,3-dihydroxyisovalerate to α-ketoisovalerate, which may be catalyzed, for example, by dihydroxyacid dehydratase;
- α-ketoisovalerate to valine, which may be catalyzed, for example, by transaminase or valine dehydrogenase;
- valine to isobutylamine, which may be catalyzed, for example, by valine decarboxylase;
- isobutylamine to isobutyraldehyde, which may be catalyzed by, for example, omega transaminase; and,
- isobutyraldehyde to isobutanol, which may be catalyzed, for example, by a branched-chain alcohol dehydrogenase.

In another embodiment, the isobutanol biosynthetic pathway comprises the following substrate to product conversions:

- pyruvate to acetolactate, which may be catalyzed, for example, by acetolactate synthase;
- acetolactate to 2,3-dihydroxyisovalerate, which may be catalyzed, for example, by acetohydroxy acid reductoisomerase;
- 2,3-dihydroxyisovalerate to α-ketoisovalerate, which may be catalyzed, for example, by acetohydroxy acid dehydratase;
- α-ketoisovalerate to isobutyryl-CoA, which may be catalyzed, for example, by branched-chain keto acid dehydrogenase;
- isobutyryl-CoA to isobutyraldehyde, which may be catalyzed, for example, by acetylating aldehyde dehydrogenase; and,
- isobutyraldehyde to isobutanol, which may be catalyzed, for example, by a branched-chain alcohol dehydrogenase.

In another embodiment, the isobutanol biosynthetic pathway comprises the substrate to product conversions shown as steps k, g, and e in FIG. **1**.

Genes and polypeptides that can be used for the substrate to product conversions described above as well as those for additional isobutanol pathways, are described in U.S. Patent Appl. Pub. No. 200710092957 and POT Pub. No. WO 2011/019894, both incorporated by reference herein. US Appl. Pub. Nos. 2011/019894, 200710092957, 2010/0081154, which are herein incorporated by reference, describe dihydroxyacid dehydratases including those from *Lactococcus lactis* and *Streptococcus mutans*. Ketoisovalerate decarboxylases include those derived from *Lactococcus lactis, Macrococcus caseolyticus* (SEQ ID NO: 542) and *Listeria grayi* (SEQ ID NO: 543). U.S. Patent Appl. Publ. No. 2009/0269823 and U.S. Appl. Publ. No. 2011/0269199, incorporated by reference, describe alcohol dehydrogenases, including those that utilize NADH as a cofactor. Alcohol dehydrogenases include

SadB from *Achromobacter xylosoxidans*. Additional alcohol dehydrogenases include horse liver ADH and *Beijerinkia indica* ADH. Alcohol dehydrogenases include those that utilize NADH as a cofactor. In one embodiment a butanol biosynthetic pathway comprises a) a ketol-acid reductoisomerase that has a K_M for NADH less than about 300 μM, less than about 100 μM, less than about 50 μM, less than about 20 or less than about 10 μM; b) an alcohol dehydrogenase that utilizes NADH as a cofactor; or c) both a) and b).

WO 2011/019894 and US Appl. Pub. Nos. 2011/019894, 2007/0092957, 2010/0081154, which are herein incorporated by reference in their entireties, describe suitable dihydroxyacid dehydratases. Methods of increasing DHAD activity are described, for example, in U.S. Patent Application Publication No. 2010/0081173 and U.S. patent application Ser. No. 13/029,558, filed Feb. 17, 2011, which are herein incorporated by reference in their entireties.

Suitable ketol-acid reductoisomerase (KARI) enzymes are described in U.S. Patent Appl. Pub. Nos. 2008/0261230 A1, 2009/0163376, 2010/0197519, 2010/0143997 and 2011/0244536, which are herein incorporated by reference in their entireties. Examples of KARIs disclosed therein are those from *Vibrio cholerae, Pseudomonas aeruginosa* PAO1, and *Pseudomonas fluorescens* PF5. In some embodiments, the KARI enzyme has a specific activity of at least about 0.1 micromoles/min/mg, at least about 0.2 micromoles/min/mg, at least about 0.3 micromoles/min/mg, at least about 0.4 micromoles/min/mg, at least about 0.5 micromoles/min/mg, at least about 0.6 micromoles/min/mg, at least about 0.7 micromoles/min/mg, at least about 0.8 micromoles/min/mg, at least about 0.9 micromoles/min/mg, at least about 1.0 micromoles/min/mg, or at least about 1.1 micromoles/min/mg. Suitable polypeptides to catalyze the substrate to product conversion acetolactate to 2,3-dihydroxyisovalerate include those that that have a KM for NADH less than about 300 μM, less than about 100 μM, less than about 50 μM, less than about 25 μM or less than about 10 μM.

In some embodiments, the KARI utilizes NADPH. Methods of measuring NADPH consumption are known in the art. For example, US Published Application No. 2008/0261230, which is herein incorporated by reference in its entirety, provides methods of measuring NADPH consumption. In some embodiments, an NADPH consumption assay is a method that measures the disappearance of the cofactor, NADPH, during the enzymatic conversion of acetolactate to α-β-dihydroxy-isovalerate at 340 nm. The activity is calculated using the molar extinction coefficient of 6220$M^{-1}cm^{-1}$ for NADPH and is reported as μmole of NADPH consumed per min per mg of total protein in cell extracts (see Aulabaugh and Schloss, Biochemistry 29: 2824-2830, 1990).

In some embodiments, the KARI is capable of utilizing NADH. In some embodiments, the KARI is capable of utilizing NADH under anaerobic conditions. KARI enzymes using NADH are described, for example, in U.S. Patent Application Publication No. 2009/0163376, which is herein incorporated by reference in its entirety.

Additional genes that can be used can be identified by one skilled in the art through bioinformatics or using methods well-known in the art.

Additionally described in U.S. Patent Application Publication No. US 2007/0092957 A1, which is incorporated by reference herein, are construction of chimeric genes and genetic engineering of bacteria and yeast for isobutanol production using the disclosed biosynthetic pathways.

Biosynthetic pathways for the production of 1-butanol that may be used include those described in U.S. Appl. Pub. No. 2008/0182308, which is incorporated herein by reference. In one embodiment, the 1-butanol biosynthetic pathway comprises the following substrate to product conversions:

a) acetyl-CoA to acetoacetyl-CoA, which may be catalyzed, for example, by acetyl-CoA acetyl transferase;
b) acetoacetyl-CoA to 3-hydroxybutyryl-CoA, which may be catalyzed, for example, by 3-hydroxybutyryl-CoA dehydrogenase;
c) 3-hydroxybutyryl-CoA to crotonyl-CoA, which may be catalyzed, for example, by crotonase;
d) crotonyl-CoA to butyryl-CoA, which may be catalyzed, for example, by butyryl-CoA dehydrogenase;
e) butyryl-CoA to butyraldehyde, which may be catalyzed, for example, by butyraldehyde dehydrogenase; and,
f) butyraldehyde to 1-butanol, which may be catalyzed, for example, by butanol dehydrogenase.

Biosynthetic pathways for the production of 2-butanol that may be used include those described in U.S. Appl. Pub. No. 2007/0259410 and U.S. Appl. Pub. No. 2009/0155870, which are incorporated herein by reference. In one embodiment, the 2-butanol biosynthetic pathway comprises the following substrate to product conversions:

a) pyruvate to alpha-acetolactate, which may be catalyzed, for example, by acetolactate synthase;
b) alpha-acetolactate to acetoin, which may be catalyzed, for example, by acetolactate decarboxylase;
c) acetoin to 3-amino-2-butanol, which may be catalyzed, for example, acetonin aminase;
d) 3-amino-2-butanol to 3-amino-2-butanol phosphate, which may be catalyzed, for example, by aminobutanol kinase;
e) 3-amino-2-butanol phosphate to 2-butanone, which may be catalyzed, for example, by aminobutanol phosphate phosphorylase; and,
f) 2-butanone to 2-butanol, which may be catalyzed, for example, by butanol dehydrogenase.

In another embodiment, the 2-butanol biosynthetic pathway comprises the following substrate to product conversions:

a) pyruvate to alpha-acetolactate, which may be catalyzed, for example, by acetolactate synthase;
b) alpha-acetolactate to acetoin, which may be catalyzed, for example, by acetolactate decarboxylase;
c) acetoin to 2,3-butanediol, which may be catalyzed, for example, by butanediol dehydrogenase;
d) 2,3-butanediol to 2-butanone, which may be catalyzed, for example, by dial dehydratase; and,
e) 2-butanone to 2-butanol, which may be catalyzed, for example, by butanol dehydrogenase.

In some embodiments of the invention, a recombinant host cell comprises a biosynthetic pathway. The biosynthetic pathway can comprise reduced or eliminated aldehyde dehydrogenase activity and an isobutanol or 1-butanol biosynthetic pathway wherein the pathway comprises the substrate to product conversion pyruvate to acetolactate. In some embodiments, a host cell comprising a biosynthetic pathway capable of converting pyruvate to acetolactate comprises a polynucleotide encoding a polypeptide having acetolactate synthase activity. For example, the biosynthetic pathway can be a butanol producing pathway or a butanediol producing pathway. The biosynthetic pathway can also be a branched-chain amino acid (e.g., leucine, isoleucine, valine) producing pathway.

In other embodiments, the recombinant host cell can comprise an isobutanol, 1-butanol, or a 2-butanol biosynthetic pathway as described herein. In some embodiments, the butanol biosynthetic pathway is an isobutanol biosynthetic pathway. Production of isobutanol or 2-butanol in a recom-

binant host cell disclosed herein may benefit from a reduction, substantial elimination or elimination of an acetolactate reductase activity.

Modifications

Functional deletion of the pyruvate decarboxylase gene has been used to increase the availability of pyruvate for utilization in biosynthetic product pathways. For example, U.S. Application Publication No. US 2007/0031950 A1, which is herein incorporated by reference in its entirety, discloses a yeast strain with a disruption of one or more pyruvate decarboxylase genes and expression of a D-lactate dehydrogenase gene, which is used for production of D-lactic acid. U.S. Application Publication No. US 2005/0059136 A1, which is herein incorporated by reference in its entirety, discloses glucose tolerant two carbon source independent (GCSI) yeast strains with no pyruvate decarboxylase activity, which can have an exogenous lactate dehydrogenase gene. Nevoigt and Stahl (*Yeast* 12:1331-1337 (1996)) describe the impact of reduced pyruvate decarboxylase and increased NAD-dependent glycerol-3-phosphate dehydrogenase in *Saccharomyces cerevisiae* on glycerol yield. U.S. Appl. Pub. No. 2009/0305363, which is herein incorporated by reference in its entirety, discloses increased conversion of pyruvate to acetolactate by engineering yeast for expression of a cytosol-localized acetolactate synthase and substantial elimination of pyruvate decarboxylase activity.

In embodiments of the invention, a recombinant host cell disclosed herein can comprise a modification in an endogenous polynucleotide encoding a polypeptide having pyruvate decarboxylase (PDC) activity or a modification in an endogenous polypeptide having PDC activity. In embodiments, a recombinant host cell disclosed herein can have a modification or disruption of a polynucleotide, gene and/or polypeptide encoding PDC. In embodiments, a recombinant host cell comprises a deletion, mutation, and/or substitution in an endogenous polynucleotide or gene encoding a polypeptide having PDC activity, or in an endogenous polypeptides having PDC activity. Such modifications, disruptions, deletions, mutations, and/or substitutions can result in PDC activity that is reduced or eliminated, resulting, for example, in a PDC knock-out (PDC-KO) phenotype.

In embodiments of the invention, an endogenous pyruvate decarboxylase activity of a recombinant host cell disclosed herein converts pyruvate to acetaldehyde, which can then be converted to ethanol or to acetyl-CoA via acetate. In other embodiments, a recombinant host cell is *Kiuyveromyces lactis* containing one gene encoding pyruvate decarboxylase, *Candida glabrata* containing one gene encoding pyruvate decarboxylase, or *Schizosaccharomyces pombe* containing one gene encoding pyruvate decarboxylase.

In other embodiments, a recombinant host cell is *Saccharomyces cerevisiae* containing three isozymes of pyruvate decarboxylase encoded by the PDC1, PDC5, and PDC6 genes, as well as a pyruvate decarboxylase regulatory gene, PDC2. In a non-limiting example in *S. cerevisiae*, the PDC1 and PDC5 genes, or the PDC1, PDC5, and PDC6 genes, are disrupted. In another non-limiting example in *S. cerevisiae*, pyruvate decarboxylase activity can be reduced by disrupting the PDC2 regulatory gene. In another non-limiting example in *S. cerevisiae*, polynucleotides or genes encoding pyruvate decarboxylase proteins such as those having about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 96%, about 97%, about 98%, or about 99% sequence identity to PDC1, PDC2, PDC5 and/or PDC6 can be disrupted.

In embodiments, a polypeptide having PDC activity or a polynucleotide or gene encoding a polypeptide having PDC activity corresponds to Enzyme Commission Number EC 4.1.1.1. In other embodiments, a PDC gene of a recombinant host cell disclosed herein is not active under the fermentation conditions used, and therefore such a gene would not need to be modified or inactivated.

Examples of recombinant host cells with reduced pyruvate decarboxylase activity due to disruption of pyruvate decarboxylase encoding genes have been reported, such as for *Saccharomyces* in Flikweert et al. (*Yeast* (1996) 12:247-257), for *Kluyveromyces* in Bianchi et al. (*Mol. Microbiol.* (1996) 19(1):27-36), and disruption of the regulatory gene in Hohmann (*Mol. Gen. Genet.* (1993) 241:657-666). *Saccharomyces* strains having no pyruvate decarboxylase activity are available from the ATCC with Accession #200027 and #200028. Examples of PDC polynucleotides, genes and/or polypeptides that can be targeted for modification or inactivation in the recombinant host cells disclosed herein include, but are not limited to, those of the following Table 8.

TABLE 8

Pyruvate decarboxylase target gene coding regions and proteins.

Description	SEQ ID NO: Nucleic acid	SEQ ID NO: Amino acid
PDC1 pyruvate decarboxylase from *Saccharomyces cerevisiae*	648	649
PDC5 pyruvate decarboxylase from *Saccharomyces cerevisiae*	650	651
PDC6 pyruvate decarboxylase from *Saccharomyces cerevisiae*	652	653
pyruvate decarboxylase from *Candida glabrata*	654	655
PDC1 pyruvate decarboxylase from *Pichia stipitis*	656	657
PDC2 pyruvate decarboxylase from *Pichia stipitis*	658	659
pyruvate decarboxylase from *Kluyveromyces lactis*	660	661
pyruvate decarboxylase from *Yarrowia lipolytica*	662	663
pyruvate decarboxylase from *Schizosaccharomyces pombe*	664	665
pyruvate decarboxylase from *Zygosaccharomyces rouxii*	666	667

Other examples of PDC polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to, PDC polynucleotides, genes and/or polypeptides having at least about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 96%, about 97%, about 98%, or about 99% sequence identity to any one of the sequences of Table 8, wherein such a polynucleotide or gene encodes, or such a polypeptide has, PDC activity. Still other examples of PDC polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to an active variant, fragment or derivative of any one of the sequences of Table 8, wherein such a polynucleotide or gene encodes, or such a polypeptide has, PDC activity.

In embodiments, a polynucleotide, gene and/or polypeptide encoding a PDC sequence disclosed herein or known in the art can be modified, as disclosed above for aldehyde dehydrogenase. In other embodiments, a polynucleotide, gene and/or polypeptide encoding PDC can be used to identify another PDC polynucleotide, gene and/or polypeptide sequence or to identify a PDC homolog in other cells, as disclosed above for acetolactate dehydrogenase. Such a PDC encoding sequence can be identified, for example, in the

literature and/or in bioinformatics databases well known to the skilled person. For example, the identification of a PDC encoding sequence in other cell types using bioinformatics can be accomplished through BLAST (as described above) searching of publicly available databases with a known PDC encoding DNA and polypeptide sequence, such as those provided herein. Identities are based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

The modification of PDC in a recombinant host cell disclosed herein to reduce or eliminate PDC activity can be confirmed using methods known in the art. For example, disruption of a particular pyruvate decarboxylase could be confirmed with PCR screening using primers external to the gene sequence, or by Southern blot using a probe designed to the pyruvate decarboxylase gene sequence. Alternatively, one could utilize analytical methods such as gas chromatography or HPLC to screen strains for decreased or eliminated production of acetaldehyde and/or ethanol.

Functional deletion of the hexokinase 2 gene has been used to reduce glucose repression and to increase the availability of pyruvate for utilization in biosynthetic pathways. For example, International Publication No. WO 2000/061722 A1, which is incorporated herein by reference in its entirety discloses the production of yeast biomass by aerobically growing yeast having one or more functionally deleted hexokinase 2 genes or analogs. In addition, Rossell et al. (Yeast Research 8:155-164 (2008)) found that *Saccharomyces cerevisiae* with a deletion of the hexokinase 2 gene showed 75% reduction in fermentative capacity, defined as the specific rate of carbon dioxide production under sugar-excess and anaerobic conditions. After starvation, the fermentation capacity was similar to that of a strain without the hexokinase 2 gene deletion. Diderich et al. (Applied and Environmental Microbiology 67:1587-1593 (2001)) found that *S. cerevisiae* with a deletion of the hexokinase 2 gene had lower pyruvate decarboxylase activity.

In embodiments, a recombinant host cell disclosed herein can comprise a modification in an endogenous polynucleotide encoding a polypeptide having hexokinase 2 activity and/or a modification in a polypeptide having hexokinase 2 activity. In embodiments, a recombinant host cell disclosed herein can have a modification or disruption of a polynucleotide, gene or polypeptide encoding hexokinase 2. In embodiments, a recombinant host cell comprises a deletion, mutation, and/or substitution in an endogenous polynucleotide or gene encoding a polypeptide having hexokinase 2 activity, or an endogenous polypeptide having hexokinase 2 activity. Such modifications, disruptions, deletions, mutations, and/or substitutions can result in hexokinase 2 activity that is reduced or eliminated, resulting, for example, in a hexokinase 2 knockout (HXK2-KO) phenotype. In embodiments, the host cell comprises a modification as described in U.S. Appn. Serial. Nos. 2011/0124060 A1 or 2012/0015416 A1, which are incorporated herein by reference in their entireties.

In embodiments, a polypeptide having hexokinase 2 activity can catalyze the conversion of hexose to hexose-6-phosphate, and/or can catalyze the conversion of D-glucose to D-glucose 6-phosphate, D-fructose to D-fructose 6-phosphate, and/or D-mannose to D-mannose 6-phosphate. In other embodiments, a polynucleotide, gene or polypeptide having hexokinase 2 activity can correspond to Enzyme Commission Number EC 2.7.1.1.

In embodiments of the invention, a recombinant host cell can be *S. cerevisiae* and a polynucleotide, gene or polypeptide having hexokinase 2 activity can be HXK2. In other embodiments, a recombinant host cell can be *K. lactis* and a polynucleotide, gene or polypeptide having hexokinase 2 activity can be RAGS. In other embodiments, a recombinant host cell can be *H. polymorpha* and a polynucleotide, gene or polypeptide having hexokinase 2 activity can be HPGLK1. In other embodiments, a recombinant host cell can be *S. pombe* and a polynucleotide, gene or polypeptide having hexokinase 2 activity can be HXK2.

Examples of hexokinase 2 polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to, those of the following Table 9.

TABLE 9

Hexokinase 2 target gene coding regions and proteins.

HXK2 from	Nucleic acid (SEQ ID NO: 668)
S. cerevisiae	Amino acid (SEQ ID NO: 669)
RAG5 from	Nucleic acid (SEQ ID NO: 670):
K. lactis	Amino acid (SEQ ID NO: 671):
HPGLK1 from	Nucleic acid (SEQ ID NO: 672)
H. polymorpha	Amino acid (SEQ ID NO: 673)
HXK2 from	Nucleic acid (SEQ ID NO: 674):
S. pombe	Amino acid (SEQ ID NO: 675):

Other examples of hexokinase 2 polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to, hexokinase 2 polynucleotides, genes and/or polypeptides having at least about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 96%, about 97%, about 98%, or about 99% sequence identity to any one of the sequences of Table 9, wherein such a polynucleotide or gene encodes, or such a polypeptide has, hexokinase 2 activity. Still other examples of hexokinase 2 polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to an active variant, fragment or derivative of any one of the sequences of Table 9, wherein such a polynucleotide or gene encodes, or such a polypeptide has, hexokinase 2 activity.

In embodiments, a polynucleotide, gene and/or polypeptide encoding a hexokinase 2 sequence disclosed herein or known in the art can be modified or disrupted, as disclosed above for aldehyde dehydrogenase. In other embodiments, a polynucleotide, gene and/or polypeptide encoding hexokinase 2 can be used to identify another hexokinase 2 polynucleotide, gene and/or polypeptide sequence or to identify a hexokinase 2 homolog in other cells, as disclosed above for aldehyde dehydrogenase. Such a hexokinase 2 encoding sequence can be identified, for example, in the literature and/or in bioinformatics databases well known to the skilled person. For example, the identification of a hexokinase 2 encoding sequence in other cell types using bioinformatics can be accomplished through BLAST (as described above) searching of publicly available databases with a known hexokinase 2 encoding DNA and polypeptide sequence, such as those provided herein. Identities are based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

The modification of hexokinase 2 in a recombinant host cell disclosed herein to reduce or eliminate hexokinase 2 activity can be confirmed using methods known in the art. For example, disruption of hexokinase 2 could be confirmed with PCR screening using primers external to the hexokinase 2

gene, or by Southern blot using a probe designed to the hexokinase 2 gene sequence. Alternatively, one could examine putative hexokinase 2 knockout strains for increased biomass yield on glucose-containing media.

Examples of additional modifications that can be useful in cells provided herein include modifications to reduce glycerol-3-phosphate dehydrogenase activity and/or disruption in at least one gene encoding a polypeptide having pyruvate decarboxylase activity or a disruption in at least one gene encoding a regulatory element controlling pyruvate decarboxylase gene expression as described in U.S. Patent Appl. Pub. No. 2009/0305363 (incorporated herein by reference), modifications to a host cell that provide for increased carbon flux through an Entner-Doudoroff Pathway or reducing equivalents balance as described in U.S. Patent Appl. Pub. No. 2010/0120105 (incorporated herein by reference). Other modifications include integration of at least one polynucleotide encoding a polypeptide that catalyzes a step in a pyruvate-utilizing biosynthetic pathway described in POT Appn. Pub. No. WO 2012/033832, which is herein incorporated by reference in its entirety. A genetic modification which has the effect of reducing glucose repression wherein the yeast production host cell is pdc- is described in U.S. Appl. Publ No. US 2011/0124060, which is herein incorporated by reference in its entirety.

U.S. Appl. Publ. No. 20120064561A1, which is herein incorporated by reference, discloses recombinant host cells comprising (a) at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity; and (b)(i) at least one deletion, mutation, and/or substitution in an endogenous gene encoding a polypeptide affecting Fe—S duster biosynthesis; and/or (ii) at least one heterologous polynucleotide encoding a polypeptide affecting Fe—S duster biosynthesis. In embodiments, the polypeptide affecting Fe—S duster biosynthesis is encoded by AFT1, AFT2, FRA2, GRX3, or CCC1. In embodiments, the polypeptide affecting Fe—S duster biosynthesis is constitutive mutant AFT1 L99A, AFT1 L102A, AFT1 C291F, or AFT1 C293F.

Additionally, host cells can comprise heterologous polynucleotides encoding a polypeptides with phosphoketolase activity and/or a heterologous polynucleotide encoding a polypeptide with phosphotransacetylase activity such as, for example, those encoded by SEQ ID NOs: 962 and 963, and as described in POT Appn. Pub. No. WO 2011/159853, which is herein incorporated by reference in its entirety.

Isobutanol and Other Products

In embodiments of the invention, methods for the production of a product of a biosynthetic pathway are provided which comprise (a) providing a recombinant host cell disclosed herein; and (b) growing the host cell under conditions whereby the product of the biosynthetic pathway is produced. In other embodiments, the product is produced as a co-product along with ethanol. In still other embodiments, the product of the biosynthetic pathway is isobutanol.

In other embodiments of the invention, the product of the biosynthetic pathway is produced at a greater yield or amount compared to the production of the same product in a recombinant host cell that does not comprise reduced or eliminated aldehyde dehydrogenase and/or aldehyde oxidase activity and/or acetolactate reductase activity. In embodiments, yield is increased by at least about 2%, at least about 5% or at least about 10%. In embodiments, this greater yield includes production at a yield of greater than about 10% of theoretical, at a yield of greater than about 20% of theoretical, at a yield of greater than about 25% of theoretical, at a yield of greater than about 30% of theoretical, at a yield of greater than about 40% of theoretical, at a yield of greater than about 50% of theoretical, at a yield of greater than about 60% of theoretical, at a yield of greater than about 70% of theoretical, at a yield of greater than about 75% of theoretical, at a yield of greater than about 80% of theoretical at a yield of greater than about 85% of theoretical, at a yield of greater than about 90% of theoretical, at a yield of greater than about 95% of theoretical, at a yield of greater than about 96% of theoretical, at a yield of greater than about 97% of theoretical, at a yield of greater than about 98% of theoretical, at a yield of greater than about 99% of theoretical, or at a yield of about 100% of theoretical. In other embodiments, the product is produced as a co-product along with ethanol. In still other embodiments, the product of the biosynthetic pathway is isobutanol.

Any product of a biosynthetic pathway that has the conversion of isobutyraldehyde to isobutyric acid as a pathway by-product can be produced with greater effectiveness in a recombinant host cell disclosed herein having the described modification of aldehyde dehydrogenase and/or aldehyde oxidase activity. A list of such products includes, but is not limited to, isobutanol.

Microbial Hosts for Isobutanol Production

Microbial hosts for isobutanol production can be selected from bacteria, cyanobacteria, filamentous fungi and yeasts. The microbial host used for butanol production should be tolerant to isobutanol so that the yield is not limited by butanol toxicity. Although butanol-tolerant mutants have been isolated from solventogenic Clostridia, little information is available concerning the butanol tolerance of other potentially useful bacterial strains. Most of the studies on the comparison of alcohol tolerance in bacteria suggest that butanol is more toxic than ethanol (de Cavalho, et al., Microsc. Res. Tech., 64: 215-22, 2004) and (Kabelitz, et al., FEMS Microbiol. Lett., 220: 223-227, 2003, Tomas, et al., J. Bacteriol., 186: 2006-2018, 2004) report that the yield of 1-butanol during fermentation in *Clostridium acetobutylicum* can be limited by 1-butanol toxicity. The primary effect of 1-butanol on *Clostridium acetobutylicum* is disruption of membrane functions (Hermann et al., Appl. Environ. Microbiol., 50: 1238-1243, 1985).

The microbial hosts selected for the production of isobutanol should be tolerant to isobutanol and should be able to convert carbohydrates to isobutanol. The criteria for selection of suitable microbial hosts include the following: intrinsic tolerance to isobutanol, high rate of glucose utilization, availability of genetic tools for gene manipulation, and the ability to generate stable chromosomal alterations.

Suitable host strains with a tolerance for isobutanol can be identified by screening based on the intrinsic tolerance of the strain. The intrinsic tolerance of microbes to isobutanol can be measured by determining the concentration of isobutanol that is responsible for 50% inhibition of the growth rate (IC_{50}) when grown in a minimal medium. The IC_{50} values can be determined using methods known in the art. For example, the microbes of interest can be grown in the presence of various amounts of isobutanol and the growth rate monitored by measuring the optical density at 600 nanometers. The doubling time can be calculated from the logarithmic part of the growth curve and used as a measure of the growth rate. The concentration of isobutanol that produces 50% inhibition of growth can be determined from a graph of the percent inhibition of growth versus the isobutanol concentration. In one embodiment, the host strain has an IC_{50} for isobutanol of greater than about 0.5%.

The microbial host for isobutanol production should also utilize glucose at a high rate. Most microbes are capable of metabolizing carbohydrates. However, certain environmental

microbes cannot metabolize carbohydrates to high efficiency, and therefore would not be suitable hosts.

The ability to genetically modify the host is essential for the production of any recombinant microorganism. The mode of gene transfer technology can be by electroporation, conjugation, transduction or natural transformation. A broad range of host conjugative plasmids and drug resistance markers are available. The cloning vectors are tailored to the host microorganisms based on the nature of antibiotic resistance markers that can function in that host.

The microbial host also has to be manipulated in order to inactivate competing pathways for carbon flow by deleting various genes. This requires the availability of either transposons to direct inactivation or chromosomal integration vectors. Additionally, the production host should be amenable to chemical mutagenesis so that mutations to improve intrinsic isobutanol tolerance can be obtained.

Based on the criteria described above, suitable microbial hosts for the production of isobutanol include, but are not limited to, members of the genera *Clostridium, Zymomonas, Escherichia, Salmonella, Rhodococcus, Pseudomonas, Bacillus, Vibrio, Lactobacillus, Enterococcus, Alcaligenes, Klebsiella, Paenibacillus, Arthrobacter, Corynebacterium, Brevibacterium, Pichia, Candida, Issatchenkia, Hansenula, Kluyveromyces*, and *Saccharomyces*. Suitable hosts include: *Escherichia coli, Alcaligenes eutrophus, Bacillus licheniformis, Paenibacillus macerans, Rhodococcus erythropolis, Pseudomonas putida, Lactobacillus plantarum, Enterococcus faecium, Enterococcus gallinarium, Enterococcus faecalis, Bacillus subtilis* and *Saccharomyces cerevisiae*. In some embodiments, the host cell is *Saccharomyces cerevisiae. S. cerevisiae* yeast are known in the art and are available from a variety of sources, including, but not limited to, American Type Culture Collection (Rockville, Md.), Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre, LeSaffre, Gert Strand AB, Ferm Solutions, North American Bioproducts, Martrex, and Lallemand. *S. cerevisiae* include, but are not limited to, BY4741, CEN.PK 113-7D, Ethanol Red® yeast, Ferm Pro™ yeast, Bio-Ferm® XR yeast, Gert Strand Prestige Batch Turbo alcohol yeast, Gert Strand Pot Distillers yeast, Gert Strand Distillers Turbo yeast, FerMax™ Green yeast, FerMax™ Gold yeast, Thermosacc® yeast, BG-1, PE-2, CAT-1, CBS7959, CBS7960, and CBS7961.

Construction of Production Host

Recombinant microorganisms containing the necessary genes that will encode the enzymatic pathway for the conversion of a fermentable carbon substrate to butanol can be constructed using techniques well known in the art. In the present invention, genes encoding the enzymes of one of the isobutanol biosynthetic pathways of the invention, for example, acetolactate synthase, acetohydroxy acid isomeroreductase, acetohydroxy acid dehydratase, branched-chain α-keto acid decarboxylase, and branched-chain alcohol dehydrogenase, can be isolated from various sources, as described above.

Methods of obtaining desired genes from a genome are common and well known in the art of molecular biology. For example, if the sequence of the gene is known, suitable genomic libraries can be created by restriction endonuclease digestion and can be screened with probes complementary to the desired gene sequence. Once the sequence is isolated, the DNA can be amplified using standard primer-directed amplification methods such as polymerase chain reaction (U.S. Pat. No. 4,683,202) to obtain amounts of DNA suitable for transformation using appropriate vectors. Tools for codon optimization for expression in a heterologous host are readily available. Some tools for codon optimization are available based on the GC content of the host microorganism.

Once the relevant pathway genes are identified and isolated they can be transformed into suitable expression hosts by means well known in the art. Vectors or cassettes useful for the transformation of a variety of host cells are common and commercially available from companies such as EPICENTRE® (Madison, Wis.), Invitrogen Corp. (Carlsbad, Calif.), Stratagene (La Jolla, Calif.), and New England Biolabs, Inc. (Beverly, Mass.). Typically the vector or cassette contains sequences directing transcription and translation of the relevant gene, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. Both control regions can be derived from genes homologous to the transformed host cell, although it is to be understood that such control regions can also be derived from genes that are not native to the specific species chosen as a production host.

Initiation control regions or promoters, which are useful to drive expression of the relevant pathway coding regions in the desired host cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving these genetic elements, including those used in the Examples, is suitable for the present invention including, but not limited to, CYC1, HIS3, GAL1, GAL10, ADH1, PGK, PHO5, GAPDH, ADC1, TRP1, URA3, LEU2, ENO, TPI (useful for expression in *Saccharomyces*); AOK1 (useful for expression in *Pichia*); and lac, ara, tet, trp, IP_L, IP_R, T7, tac, and trc (useful for expression in *Escherichia coli, Alcaligenes*, and *Pseudomonas*) as well as the amy, apr, npr promoters and various phage promoters useful for expression in *Bacillus subtilis, Bacillus licheniformis*, and *Paenibacillus macerans*. For yeast recombinant host cells, a number of promoters can be used in constructing expression cassettes for genes, including, but not limited to, the following constitutive promoters suitable for use in yeast: FBA1, TDH3 (GPD), ADH1, ILV5, and GPM1; and the following inducible promoters suitable for use in yeast: GAL1, GAL10, OLE1, and CUP1. Other yeast promoters include hybrid promoters UAS(PGK1)-FBA1p (SEQ ID NO: 406), UAS(PGK1)-ENO2p (SEQ ID NO: 538), UAS(FBA1)-PDC1p (SEQ ID NO: 539), UAS (PGK1)-PDC1p (SEQ ID NO: 540), and UAS(PGK)-OLE1p (SEQ ID NO: 541).

Promoters, transcriptional terminators, and coding regions can be cloned into a yeast 2 micron plasmid and transformed into yeast cells (Ludwig, et al, Gene, 132: 33-40, 1993; US Appl. Pub. No. 20080261861 A1).

Adjusting the amount of gene expression in a given host may be achieved by varying the level of transcription, such as through selection of native or artificial promoters. In addition, techniques such as the use of promoter libraries to achieve desired levels of gene transcription are well known in the art. Such libraries can be generated using techniques known in the art, for example, by cloning of random cDNA fragments in front of gene cassettes (Goh et al. (2002) *AEM* 99, 17025), by modulating regulatory sequences present within promoters (Ligr et al. (2006) *Genetics* 172, 2113), or by mutagenesis of known promoter sequences (Alper et al. (2005) *PNAS,* 12678; Nevoigt et al. (2006) *AEM* 72, 5266).

Termination control regions can also be derived from various genes native to the hosts. Optionally, a termination site can be unnecessary or can be included.

Certain vectors are capable of replicating in a broad range of host bacteria and can be transferred by conjugation. The complete and annotated sequence of pRK404 and three

related vectors-pRK437, pRK442, and pRK442(H) are available. These derivatives have proven to be valuable tools for genetic manipulation in Gram-negative bacteria (Scott et al., Plasmid, 50: 74-79, 2003), Several plasmid derivatives of broad-host-range Inc P4 plasmid RSF1010 are also available with promoters that can function in a range of Gram-negative bacteria, Plasmid pAYC36 and pAYC37, have active promoters along with multiple cloning sites to allow for the heterologous gene expression in Gram-negative bacteria.

Chromosomal gene replacement tools are also widely available. For example, a thermosensitive variant of the broad-host-range replicon pWV101 has been modified to construct a plasmid pVE6002 which can be used to effect gene replacement in a range of Gram-positive bacteria (Maguin et al., J. Bacteriol., 174: 5633-5638, 1992). Additionally, in vitro transposomes are available to create random mutations in a variety of genomes from commercial sources such as EPICENTRE®.

The expression of a butanol biosynthetic pathway in various microbial hosts is described in more detail below.

Expression of a Butanol Biosynthetic Pathway in *E. Coli*

Vectors or cassettes useful for the transformation of *E. coli* are common and commercially available from the companies listed above. For example, the genes of an isobutanol biosynthetic pathway can be isolated from various sources, cloned into a modified pUC19 vector and transformed into *E. coli* NM522.

Expression of a Butanol Biosynthetic Pathway in *Rhodococcus erythropolis*

A series of *E. coli-Rhodococcus* shuttle vectors are available for expression in *R. erythropolis*, including, but not limited to, pRhBR17 and pDA71 (Kostichka et al., Appl. Microbiol. Biotechnol., 62: 61-68, 2003). Additionally, a series of promoters are available for heterologous gene expression in *R. erythropolis* (Nakashima et al., Appl. Environ. Microbiol., 70: 5557-5568, 2004 and Tao et al., Appl. Microbiol. Biotechnol., 68: 346-354, 2005). Targeted gene disruption of chromosomal genes in *R. erythropolis* can be created using the method described by Tao et al., supra, and Brans et al. (Appl. Environ. Microbiol., 66: 2029-2036, 2000).

The heterologous genes required for the production of isobutanol, as described above, can be cloned initially in pDA71 or pRhBR71 and transformed into *E. coli*. The vectors can then be transformed into *R. erythropolis* by electroporation, as described by Kostichka et al., supra. The recombinants can be grown in synthetic medium containing glucose and the production of isobutanol can be followed using methods known in the art.

Expression of a Butanol Biosynthetic Pathway in *B. Subtilis*

Methods for gene expression and creation of mutations in *B. subtilis* are also well known in the art. For example, the genes of an isobutanol biosynthetic pathway can be isolated from various sources, cloned into a modified pUC19 vector and transformed into *Bacillus subtilis* BE1010. Additionally, the five genes of an isobutanol biosynthetic pathway can be split into two operons for expression. The three genes of the pathway (bubB, ilvD, and kivD) can be integrated into the chromosome of *Bacillus subtilis* BE1010 (Payne, et al., J. Bacteriol., 173, 2278-2282, 1991). The remaining two genes (ilvC and bdhB) can be cloned into an expression vector and transformed into the *Bacillus* strain carrying the integrated isobutanol genes

Expression of a Butanol Biosynthetic Pathway in *B. licheniformis*

Most of the plasmids and shuttle vectors that replicate in *B. subtilis* can be used to transform *B. licheniformis* by either protoplast transformation or electroporation. The genes required for the production of isobutanol can be cloned in plasmids pBE20 or pBE60 derivatives (Nagarajan et al., Gene, 114: 121-126, 1992). Methods to transform *B. licheniformis* are known in the art (Fleming et al. Appl. Environ, Microbiol., 61: 3775-3780, 1995). The plasmids constructed for expression in *B. subtilis* can be transformed into *B. licheniformis* to produce a recombinant microbial host that produces isobutanol.

Expression of a Butanol Biosynthetic Pathway in *Paenibacillus macerans*

Plasmids can be constructed as described above for expression in *B. subtilis* and used to transform *Paenibacillus macerans* by protoplast transformation to produce a recombinant microbial host that produces isobutanol.

Expression of the Butanol Biosynthetic Pathway in *Alcaligenes* (*Ralstonia*) *eutrophus*

Methods for gene expression and creation of mutations in *Alcaligenes eutrophus* are known in the art (Taghavi et al., Appl. Environ, Microbiol., 60: 3585-3591, 1994). The genes for an isobutanol biosynthetic pathway can be cloned in any of the broad host range vectors described above, and electroporated to generate recombinants that produce isobutanol. The poly(hydroxybutyrate) pathway in *Alcaligenes* has been described in detail, a variety of genetic techniques to modify the *Alcaligenes* eutrophus genome is known, and those tools can be applied for engineering an isobutanol biosynthetic pathway.

Expression of a Butanol Biosynthetic Pathway in *Pseudomonas putida*

Methods for gene expression in *Pseudomonas putida* are known in the art (see for example Ben-Basset et al., U.S. Pat. No. 6,586,229, which is incorporated herein by reference). The butanol pathway genes can be inserted into pPCU18 and this ligated DNA can be electroporated into electrocompetent *Pseudomonas putida* DOT-T1 C5aAR1 cells to generate recombinants that produce isobutanol.

Expression of a Butanol Biosynthetic Pathway in *Saccharomyces cerevisiae*

Methods for gene expression in *Saccharomyces cerevisiae* are known in the art (e.g., *Methods in Enzymology*, Volume 194, *Guide to Yeast Genetics and Molecular and Cell Biology*, Part A, 2004, Christine Guthrie and Gerald R. Fink, eds., Elsevier Academic Press, San Diego, Calif.). Expression of genes in yeast typically requires a promoter, followed by the gene of interest, and a transcriptional terminator. A number of yeast promoters, including those used in the Examples herein, can be used in constructing expression cassettes for genes encoding an isobutanol biosynthetic pathway, including, but not limited to constitutive promoters FBA, GPD, ADH1, and GPM, and the inducible promoters GAL1, GAL10, and CUP1. Suitable transcriptional terminators include, but are not limited to FBAt, GPDt, GPMt, ERG10t, GAL1t, CYC1, and ADH1. For example, suitable promoters, transcriptional terminators, and the genes of an isobutanol biosynthetic pathway can be cloned into *E. coli*-yeast shuttle vectors and transformed into yeast cells as described in U.S. App. Pub. No. 20100129886. These vectors allow strain propagation in both *E. coli* and yeast strains. Typically the vector contains a selectable marker and sequences allowing autonomous replication or chromosomal integration in the desired host. Typically used plasmids in yeast are shuttle vectors pRS423, pRS424, pRS425, and pRS426 (American Type Culture Collection, Rockville, Md.), which contain an *E. coli* replication origin (e.g., pMB1), a yeast 2μ origin of replication, and a marker for nutritional selection. The selection markers for these four vectors are His3 (vector pRS423), Trp1 (vector pRS424), Leu2 (vector pRS425) and Ura3 (vector pRS426).

Construction of expression vectors with genes encoding polypeptides of interest can be performed by either standard molecular cloning techniques in *E. coli* or by the gap repair recombination method in yeast.

The gap repair cloning approach takes advantage of the highly efficient homologous recombination in yeast. Typically, a yeast vector DNA is digested (e.g., in its multiple cloning site) to create a "gap" in its sequence. A number of insert DNAs of interest are generated that contain a ≥21 bp sequence at both the 5' and the 3' ends that sequentially overlap with each other, and with the 5' and 3' terminus of the vector DNA. For example, to construct a yeast expression vector for "Gene X", a yeast promoter and a yeast terminator are selected for the expression cassette. The promoter and terminator are amplified from the yeast genomic DNA, and Gene X is either PCR amplified from its source organism or obtained from a cloning vector comprising Gene X sequence. There is at least a 21 bp overlapping sequence between the 5' end of the linearized vector and the promoter sequence, between the promoter and Gene X, between Gene X and the terminator sequence, and between the terminator and the 3' end of the linearized vector. The "gapped" vector and the insert DNAs are then co-transformed into a yeast strain and plated on the medium containing the appropriate compound mixtures that allow complementation of the nutritional selection markers on the plasmids. The presence of correct insert combinations can be confirmed by PCR mapping using plasmid DNA prepared from the selected cells. The plasmid DNA isolated from yeast (usually low in concentration) can then be transformed into an *E. coli* strain, e.g. TOP10, followed by mini preps and restriction mapping to further verify the plasmid construct. Finally the construct can be verified by sequence analysis.

Like the gap repair technique, integration into the yeast genome also takes advantage of the homologous recombination system in yeast. Typically, a cassette containing a coding region plus control elements (promoter and terminator) and auxotrophic marker is PCR-amplified with a high-fidelity DNA polymerase using primers that hybridize to the cassette and contain 40-70 base pairs of sequence homology to the regions 5° and 3° of the genomic area where insertion is desired. The PCR product is then transformed into yeast and plated on medium containing the appropriate compound mixtures that allow selection for the integrated auxotrophic marker. For example, to integrate "Gene X" into chromosomal location "Y", the promoter-coding regionX-terminator construct is PCR amplified from a plasmid DNA construct and joined to an autotrophic marker (such as URA3) by either SOE PCR or by common restriction digests and cloning. The full cassette, containing the promoter-coding regionX-terminator-URA3 region, is PCR amplified with primer sequences that contain 40-70 bp of homology to the regions 5' and 3' of location "Y" on the yeast chromosome. The PCR product is transformed into yeast and selected on growth media lacking uracil. Transformants can be verified either by colony PCR or by direct sequencing of chromosomal DNA.

Expression of a Butanol Biosynthetic Pathway in *Lactobacillus plantarum*

The *Lactobacillus* genus belongs to the Lactobacillales family and many plasmids and vectors used in the transformation of *Bacillus subtilis* and *Streptococcus* can be used for *Lactobacillus*. Non-limiting examples of suitable vectors include pAMβ1 and derivatives thereof (Renault et al., Gene 183:175-182, 1996); and (O'Sullivan et al., Gene, 137: 227-231, 1993); pMBB1 and pHW800, a derivative of pMBB1 (Wyckoff et al., Appl. Environ. Microbiol., 62: 1481-1486, 1996); pMG1, a conjugative plasmid (Tanimoto et al., J. Bacteriol., 184: 5800-5804, 2002); pNZ9520 (Kleerebezem et al., Appl. Environ. Microbiol., 63: 4581-4584, 1997); pAM401 (Fujimoto et al., Appl. Environ. Microbiol., 67: 1262-1267, 2001); and pAT392 (Arthur et al., Antimicrob. Agents Chemother., 38: 1899-1903, 1994). Several plasmids from *Lactobacillus plantarum* have also been reported (van Kranenburg R, et al. Appl. Environ. Microbiol., 71: 1223-1230, 2005).

Expression of a Butanol Biosynthetic Pathway in Various *Enterococcus* sieges (*E. faecium, E. gallinarium*, and *E. faecalis*)

The *Enterococcus* genus belongs to the Lactobacillales family and many plasmids and vectors used in the transformation of Lactobacilli, Bacilli and Streptococci species can be used for *Enterococcus* species. Non-limiting examples of suitable vectors include pAMβ1 and derivatives thereof (Renault et al., Gene, 183: 175-182, 1996); and (O'Sullivan et al., Gene, 137: 227-231, 1993); pMBB1 and pHW800, a derivative of pMBB1 (Wyckoff et al. Appl. Environ. Microbiol., 62: 1481-1486, 1996); pMG1, a conjugative plasmid (Tanimoto et al., J. Bacteriol., 184: 5800-5804, 2002); pNZ9520 (Kleerebezem et al., Appl. Environ. Microbiol., 63: 4581-4584, 1997); pAM401 (Fujimoto et al., Appl. Environ. Microbiol., 67: 1262-1267, 2001); and pAT392 (Arthur et al., Antimicrob. Agents Chemother., 38: 1899-1903, 1994). Expression vectors for *E. faecalis* using the nisA gene from *Lactococcus* can also be used (Eichenbaum et al., Appl. Environ. Microbiol., 64: 2763-2769, 1998). Additionally, vectors for gene replacement in the *E. faecium* chromosome can be used (Nallaapareddy et al., Appl. Environ. Microbiol., 72: 334-345, 2006).

Fermentation Media

Fermentation media in the present invention must contain suitable carbon substrates. Suitable substrates can include but are not limited to monosaccharides such as glucose and fructose, oligosaccharides such as lactose, maltose, galactose, sucrose, polysaccharides such as starch or cellulose or mixtures thereof and unpurified mixtures from renewable feedstocks such as cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. Additionally the carbon substrate can also be one-carbon substrates such as carbon dioxide, or methanol for which metabolic conversion into key biochemical intermediates has been demonstrated. In addition to one and two carbon substrates methylotrophic microorganisms are also known to utilize a number of other carbon containing compounds such as methylamine, glucosamine and a variety of amino acids for metabolic activity. For example, methylotrophic yeast are known to utilize the carbon from methylamine to form trehalose or glycerol (Bellion et al., *Microb. Growth C*1 *Compd*., [Int. Symp.], 7th (1993), 415-32. (eds): Murrell, J. Collin; Kelly, Don P. Publisher; Intercept, Andover, UK). Similarly, various species of *Candida* will metabolize alanine or oleic acid (Sulter et al., Arch. Microbiol., 153: 485-489, 1990). Hence it is contemplated that the source of carbon utilized in the present invention can encompass a wide variety of carbon containing substrates and will only be limited by the choice of microorganism.

Although it is contemplated that all of the above mentioned carbon substrates and mixtures thereof are suitable in the present invention, in some embodiments, the carbon substrates are glucose, fructose, and sucrose, or mixtures of these with C5 sugars such as xylose and/or arabinose for yeasts cells modified to use C5 sugars. Sucrose can be derived from renewable sugar sources such as sugar cane, sugar beets, cassava, sweet sorghum, and mixtures thereof. Glucose and dextrose can be derived from renewable grain sources through saccharification of starch based feedstocks including

grains such as corn, wheat, rye, barley, oats, and mixtures thereof. In addition, fermentable sugars can be derived from renewable cellulosic or lignocellulosic biomass through processes of pretreatment and saccharification, as described, for example, in U.S. Parent App. Pub. No. 2007/0031918 A1, which is herein incorporated by reference in its entirety. Biomass refers to any cellulosic or lignocellulosic material and includes materials comprising cellulose, and optionally further comprising hemicellulose, lignin, starch, oligosaccharides and/or monosaccharides. Biomass can also comprise additional components, such as protein and/or lipid. Biomass can be derived from a single source, or biomass can comprise a mixture derived from more than one source; for example, biomass can comprise a mixture of corn cobs and corn stover, or a mixture of grass and leaves. Biomass includes, but is not limited to, bioenergy crops, agricultural residues, municipal solid waste, industrial solid waste, sludge from paper manufacture, yard waste, wood and forestry waste. Examples of biomass include, but are not limited to, corn grain, corn cobs, crop residues such as corn husks, corn stover, grasses, wheat, wheat straw, barley, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum, soy, components obtained from milling of grains, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, flowers, animal manure, and mixtures thereof.

In addition to an appropriate carbon source, fermentation media must contain suitable minerals, salts, cofactors, buffers and other components, known to those skilled in the art, suitable for growth of the cultures and promotion of the enzymatic pathway necessary for butanol production described herein.

Culture Conditions

Typically cells are grown at a temperature in the range of about 20° C. to about 40° C. in an appropriate medium. Suitable growth media in the present invention are common commercially prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth or Yeast Medium (YM) broth or broth that includes yeast nitrogen base, ammonium sulfate, and dextrose (as the carbon/energy source) or YPD Medium, a blend of peptone, yeast extract, and dextrose in optimal proportions for growing most *Saccharomyces cerevisiae* strains. Other defined or synthetic growth media can also be used, and the appropriate medium for growth of the particular microorganism will be known by one skilled in the art of microbiology or fermentation science. The use of agents known to modulate catabolite repression directly or indirectly, e.g., cyclic adenosine 2',3'-monophosphate (CAMP), can also be incorporated into the fermentation medium.

Suitable pH ranges for the fermentation are between pH 5.0 to pH 9.0, where pH 6.0 to pH 8.0 is preferred for the initial condition. Suitable pH ranges for the fermentation of yeast are typically between about pH 3.0 to about pH 9.0. In one embodiment, about pH 5.0 to about pH 8.0 is used for the initial condition. Suitable pH ranges for the fermentation of other microorganisms are between about pH 3.0 to about pH 7.5. In one embodiment, about pH 4.5 to about pH 6.5 is used for the initial condition.

Fermentations can be performed under aerobic or anaerobic conditions. In one embodiment, anaerobic or microaerobic conditions are used for fermentation.

Industrial Batch and Continuous Fermentations

The present processes may employ a batch method of fermentation. A classical batch fermentation is a dosed system where the composition of the medium is set at the beginning of the fermentation and not subject to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the medium is inoculated with the desired microorganism or microorganisms, and fermentation is permitted to occur without adding anything to the system. Typically, however, a "batch" fermentation is batch with respect to the addition of carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. In batch systems the metabolite and biomass compositions of the system change constantly up to the time the fermentation is stopped. Within batch cultures cells moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase will eventually die. Cells in log phase generally are responsible for the bulk of production of end product or intermediate.

A variation on the standard batch system is the Fed-Batch system. Fed-Batch fermentation processes are also suitable in the present invention and comprise a typical batch system with the exception that the substrate is added in increments as the fermentation progresses. Fed-Batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium. Measurement of the actual substrate concentration in Fed-Batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases such as CO_2. Batch and Fed-Batch fermentations are common and well known in the art and examples can be found in Thomas D. Brock in *Biotechnology: A Textbook of Industrial Microbiology*, Second Edition (1989) Sinauer Associates, Inc., Sunderland, Mass., or Deshpande, Mukund (Appl. Biochem. Biotechnol., 36: 227, 1992), herein incorporated by reference.

Although the present invention is performed in batch mode it is contemplated that the method would be adaptable to continuous fermentation methods. Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor and an equal amount of conditioned medium is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth.

Continuous fermentation allows for modulation of one factor or any number of factors that affect cell growth or end product concentration. For example, one method will maintain a limiting nutrient such as the carbon source or nitrogen level at a fixed rate and allow all other parameters to moderate. In other systems a number of factors affecting growth can be altered continuously while the cell concentration, measured by medium turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions and thus the cell loss due to the medium being drawn off must be balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology and a variety of methods are detailed by Brock, supra.

It is contemplated that the present invention can be practiced using either batch, fed-batch or continuous processes and that any known mode of fermentation would be suitable. Additionally, it is contemplated that cells can be immobilized on a substrate as whole cell catalysts and subjected to fermentation conditions for isobutanol production.

Methods for Butanol Isolation from the Fermentation Medium

Bioproduced butanol can be isolated from the fermentation medium using methods known in the art for ABE fermenta-

tions (see, e.g., Durre, *Appl. Microbiol. Biotechnol.* 49:639-648 (1998), Groot et al., *Process. Biochem.* 27:61-75 (1992), and references therein). For example, solids can be removed from the fermentation medium by centrifugation, filtration, decantation, or the like. Then, the butanol can be isolated from the fermentation medium using methods such as distillation, azeotropic distillation, liquid-liquid extraction, adsorption, gas stripping, membrane evaporation, or pervaporation.

Because butanol forms a low boning point, azeotropic mixture with water, distillation can be used to separate the mixture up to its azeotropic composition. Distillation can be used in combination with another separation method to obtain separation around the azeotrope. Methods that can be used in combination with distillation to isolate and purify butanol include, but are not limited to, decantation, liquid-liquid extraction, adsorption, and membrane-based techniques. Additionally, butanol can be isolated using azeotropic distillation using an entrainer (see, e.g., Doherty and Malone, *Conceptual Design of Distillation Systems*, McGraw Hill, New York, 2001).

The butanol-water mixture forms a heterogeneous azeotrope so that distillation can be used in combination with decantation to isolate and purify the butanol. In this method, the butanol containing fermentation broth is distilled to near the azeotropic composition. Then, the azeotropic mixture is condensed, and the butanol is separated from the fermentation medium by decantation. The decanted aqueous phase can be returned to the first distillation column as reflux. The butanol-rich decanted organic phase can be further purified by distillation in a second distillation column.

The butanol can also be isolated from the fermentation medium using liquid-liquid extraction in combination with distillation. In this method, the butanol is extracted from the fermentation broth using liquid-liquid extraction with a suitable solvent. The butanol-containing organic phase is then distilled to separate the butanol from the solvent.

Distillation in combination with adsorption can also be used to isolate butanol from the fermentation medium. In this method, the fermentation broth containing the butanol is distilled to near the azeotropic composition and then the remaining water is removed by use of an adsorbent, such as molecular sieves (Aden et al., *Lignocellulosic Biomass to Ethanol Process Design and Economics Utilizing Co-Current Dilute Acid Prehydrolysis and Enzymatic Hydrolysis for Corn Stover*, Report NREL/TP-510-32438, National Renewable Energy Laboratory, June 2002).

Additionally, distillation in combination with pervaporation can be used to isolate and purify the butanol from the fermentation medium. In this method, the fermentation broth containing the butanol is distilled to near the azeotropic composition, and then the remaining water is removed by pervaporation through a hydrophilic membrane (Guo et al., *J. Membr. Sci.* 245, 199-210 (2004)).

In situ product removal (ISPR) (also referred to as extractive fermentation) can be used to remove butanol (or other fermentative alcohol) from the fermentation vessel as it is produced, thereby allowing the microorganism to produce butanol at high yields. One method for ISPR for removing fermentative alcohol that has been described in the art is liquid-liquid extraction. In general, with regard to butanol fermentation, for example, the fermentation medium, which includes the microorganism, is contacted with an organic extractant at a time before the butanol concentration reaches a toxic level. The organic extractant and the fermentation medium form a biphasic mixture. The butanol partitions into the organic extractant phase, decreasing the concentration in the aqueous phase containing the microorganism, thereby limiting the exposure of the microorganism to the inhibitory butanol.

Liquid-liquid extraction can be performed, for example, according to the processes described in U.S. Patent Appl. Pub. No. 2009/0305370, the disclosure of which is hereby incorporated in its entirety. U.S. Patent Appl. Pub. No. 2009/0305370 describes methods for producing and recovering butanol from a fermentation broth using liquid-liquid extraction, the methods comprising the step of contacting the fermentation broth with a water immiscible extractant to form a two-phase mixture comprising an aqueous phase and an organic phase. Typically, the extractant can be an organic extractant selected from the group consisting of saturated, mono-unsaturated, poly-unsaturated (and mixtures thereof) C_{12} to C_{22} fatty alcohols, C_{12} to C_{22} fatty acids, esters of C_{12} to C_{22} fatty acids, C_{12} to C_{22} fatty aldehydes, and mixtures thereof. The extractant(s) for ISPR can be non-alcohol extractants. The ISPR extractant can be an exogenous organic extractant such as oleyl alcohol, behenyl alcohol, cetyl alcohol, lauryl alcohol, myristyl alcohol, stearyl alcohol, 1-undecanol, oleic acid, lauric acid, myristic acid, stearic acid, methyl myristate, methyl oleate, undecanal, lauric aldehyde, 20-methylundecanal, and mixtures thereof.

In some embodiments, the ester can be formed by contacting the alcohol in a fermentation medium with a carboxylic acid (e.g., fatty acids) and a catalyst capable of esterifying the alcohol with the carboxylic acid, as described in POT Appn, Pub. No. WO/2011/159998, which is herein incorporated by reference in its entirety. In such embodiments, the carboxylic acid can serve as an ISPR extractant into which the alcohol esters partition. The carboxylic acid can be supplied to the fermentation vessel and/or derived from the biomass supplying fermentable carbon fed to the fermentation vessel. Lipids present in the feedstock can be catalytically hydrolyzed to carboxylic acid, and the same catalyst (e.g., enzymes) can esterify the carboxylic acid with the alcohol. The catalyst can be supplied to the feedstock prior to fermentation, or can be supplied to the fermentation vessel before or contemporaneously with the supplying of the feedstock. When the catalyst is supplied to the fermentation vessel, alcohol esters can be obtained by hydrolysis of the lipids into carboxylic acid and substantially simultaneous esterification of the carboxylic acid with butanol present in the fermentation vessel. Carboxylic acid and/or native oil not derived from the feedstock can also be fed to the fermentation vessel, with the native oil being hydrolyzed into carboxylic acid. Any carboxylic acid not esterified with the alcohol can serve as part of the ISPR extractant. The extractant containing alcohol esters can be separated from the fermentation medium, and the alcohol can be recovered from the extractant. The extractant can be recycled to the fermentation vessel. Thus, in the case of butanol production, for example, the conversion of the butanol to an ester may reduce the free butanol concentration in the fermentation medium, shielding the microorganism from the toxic effect of increasing butanol concentration. In addition, unfractionated grain can be used as feedstock without separation of lipids therein, since the lipids can be catalytically hydrolyzed to carboxylic acid, thereby decreasing the rate of build-up of lipids in the ISPR extractant.

In situ product removal can be carried out in a batch mode or a continuous mode. In a continuous mode of in situ product removal, product is continually removed from the reactor. In a batchwise mode of in situ product removal, a volume of organic extractant is added to the fermentation vessel and the extractant is not removed during the process. For in situ product removal, the organic extractant can contact the fer-

mentation medium at the start of the fermentation forming a biphasic fermentation medium. Alternatively, the organic extractant can contact the fermentation medium after the microorganism has achieved a desired amount of growth, which can be determined by measuring the optical density of the culture. Further, the organic extractant can contact the fermentation medium at a time at which the product alcohol level in the fermentation medium reaches a preselected level. In the case of butanol production according to some embodiments of the present invention, the carboxylic acid extractant can contact the fermentation medium at a time before the butanol concentration reaches a toxic level, so as to esterify the butanol with the carboxylic acid to produce butanol esters and consequently reduce the concentration of butanol in the fermentation vessel. The ester-containing organic phase can then be removed from the fermentation vessel (and separated from the fermentation broth which constitutes the aqueous phase) after a desired effective titer of the butanol esters is achieved. In some embodiments, the ester-containing organic phase is separated from the aqueous phase after fermentation of the available fermentable sugar in the fermentation vessel is substantially complete.

EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various uses and conditions.

General Methods:

Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, by T. J. Silhavy, M. L. Bennan, and L. W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1984, and by Ausubel, F. M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience, N.Y., 1987.

Materials and methods suitable for the maintenance and growth of bacterial cultures are also well known in the art. Techniques suitable for use in the following Examples can be found in *Manual of Methods for General Bacteriology*, Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds., American Society for Microbiology, Washington, D.C., 1994, or by Thomas D. Brock in *Biotechnology: A Textbook of Industrial Microbiology*, Second Edition, Sinauer Associates, Inc., Sunderland, Mass., 1989. All reagents, restriction enzymes and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, Wis.), BD Diagnostic Systems (Sparks, Md.), Life Technologies (Rockville, Md.), or Sigma Chemical Company (St. Louis, Mo.), unless otherwise specified.

The meaning of abbreviations used is as follows: "Å" means Angstrom, "min" means minute(s), "h" means hour(s), "μl" means microliter(s), "ng/μl" means nano gram per microliter, "pmol/μl" means pico mole per microliter, "ml" means milliliter(s), "L" means liter(s), "g/L" mean gram per liter, "ng" means nano gram, "sec" means second(s), "ml/min" means milliliter per minute(s), "w/v" means weight per volume, "v/v" means volume per volume, "nm" means nanometer(s), "mm" means millimeter(s), "cm" means centimeter(s), "mM" means millimolar, "M" means molar, "g" means gram(s), "μg" means microgram(s), "mg" means milligram(s), "g" means the gravitation constant, "rpm" means revolutions per minute, "HPLC" means high performance liquid chromatography, "MS" means mass spectrometry, "HPLC/MS" means high performance liquid chromatography/mass spectrometry, "EDTA" means ethylendiamine-tetraacetic acid, "dNTP" means deoxynucleotide triphosphate, "° C." means degrees Celsius, and "V" means voltage.

High Throughput Screening Assay of Gene Libraries

High throughput screening of the gene libraries of mutant KARI enzymes was performed as described herein (with the exception of Examples 16 and 21): 10× freezing medium containing 554.4 g/L glycerol, 68 mM of $(NH_4)_2SO_4$, 4 mM $MgSO_4$, 17 mM sodium citrate, 132 mM KH_2PO_4, 36 mM K_2HPO_4 was prepared with molecular pure water and filter-sterilized. Freezing medium was prepared by diluting the 10× freezing medium with the LB medium. An aliquot (200 μl) of the freezing medium was used for each well of the 96-well archive plates (cat #3370, Corning Inc. Corning, N.Y.).

Clones from the LB agar plates were selected and inoculated into the 96-well archive plates containing the freezing medium and grown overnight at 37° C. without shaking. The archive plates were then stored at −80° C. *E. coli* strain Bw25113 transformed with pBAD-HisB (Invitrogen) was always used as the negative control. The positive controls for the libraries in Examples 3, 4, and 5 are the wild type K9-KARI, AB1D3, AB1D3 respectively.

Clones from archive plates were inoculated into the 96-deep well plates. Each well contained 3.0 μl of cells from thawed archive plates, 200 μl of the LB medium containing 100 μg/ml ampicillin and 0.02% (w/v) arabinose as the inducer. Cells were the grown overnight at 37° C. with 80% humidity while shaking (900 rpm), harvested by centrifugation (4000 rpm, 5 min at 25° C.). (Eppendorf centrifuge, Brinkmann Instruments, Inc. Westbury, N.Y.) and the cell pellet was stored at −20° C. for later analysis. The assay substrate, (R,S)-acetolactate, was synthesized as described by Aulabaugh and Schloss (Aulabaugh and Schloss, Biochemistry, 29: 2824-2830, 1990). AH other chemicals used in the assay were purchased from Sigma.

The enzymatic conversion of acetolactate to α,β-dihydroxy-isovalerate by KARI was followed by measuring the disappearance of the cofactor, NADPH or NADH, from the reaction at 340 nm using a plate reader (Molecular Device, Sunnyvale, Calif.). The activity was calculated using the molar extinction coefficient of 6220 $M^{-1}cm^{-1}$ for either NADPH or NADH. The stock solutions used were: K_2HPO_4 (0.2 M); KH_2PO_4 (0.2 M); EDTA (0.5 M); $MgCl_2$ (1.0 M); NADPH (2.0 mM); NADH (2.0 mM) and acetolactate (45 mM). The 100 ml reaction buffer (pH 6.8) containing: 2.0 ml K_2HPO_4, 3.0 ml KH_2PO_4, 4.0 ml $MgCl_2$, 0.1 ml EDTA and 90.9 ml water was prepared.

Frozen cell pellet in deep-well plates and BugBuster were warmed up at room temperature for 30 min at the same time. Each well of 96-well assay plates was filled with 120 μl of the reaction buffer and 20 μl of NADH (2.0 mM). 75 μl of 50% BugBuster (v/v in water) was added to each well after 30 min warm-up and cells were suspended using plate shaker. The plates were incubated at room temperature for 20 min. An aliquot (15 to 25 μl depending the expected activity) of cell lysate was transferred into each well of 96-well assay plates. Absorbance at 340 nm was recorded as background, 16 μl of acetolactate (4.5 mM, diluted with the reaction buffer) was added to each well and mixed with shaking by the plate

reader. Absorbance at 340 nm was recorded at 0, and 10 to 30 minutes depending the expected activity after substrate addition. The difference in absorbance (before and after substrate addition) was used to determine the activity of the mutants. Mutants with higher KARI activity compared to the positive control were selected for re-screening.

The number of clones screened for the libraries in Example 1, 2 and 3 are about 12,000, 12,000 and 92 respectively. The top performers from each library were re-screened described below as secondary assay.

Secondary Assay of Active Mutants

Cells containing selected mutants identified by high throughput screening (above) were grown overnight, at 37° C., in 3.0 ml of the LB medium containing 100 ampicillin and 0.025% (w/v) arabinose as the inducer while shaking at 250 rpm. The cells were then aliquoted into 96 deepwell plates (200 μl per well) and harvested by centrifugation at 4,000×g for 5 min at room temperature. 75 μl of 50% BugBuster (v/v in water) was added to each well and cells were suspended using plate shaker. The plates were incubated at room temperature for 20 min. An aliquot (15 to 25 μl depending the expected activity) of cell lysate was transferred into each well of 96-well assay plates, which contain 120 μl of the reaction buffer and 20 μl of NADH (2.0 mM) per well. Absorbance at 340 nm was recorded as background, 16 μl of acetolactate (4.5 mM, diluted with the reaction buffer) was added to each well and mixed with shaking by the plate reader. Absorbance at 340 nm was recorded at 0, and 5 to 10 minutes depending the expected activity after substrate addition. The difference in absorbance (before and after substrate addition) was used to determine the activity of the mutants. Mutants with higher KARI activity compared to the positive control were selected for further characterization.

Measurement of NADH and NADPH Michaelis Constants

KARI enzyme activity can be routinely measured by NADH or NADPH oxidation as described above, however to measure the Michaelis constant (K_M) for these pyridine nucleotides formation of the 2,3-dihydroxyisovalerate product was measured directly using HPLC/MS.

Protein concentration of crude cell extract from Bugbuster lysed cells (as described above) was measured using the Bio-Rad protein assay reagent (BioRad Laboratories, Inc., Hercules, Calif. 94547). Between 0.2 and 1.0 micrograms of crude extract protein was added to a reaction buffer consisting of 100 mM MOPS KOH, pH 6.8, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM glucose-6-phosphate (Sigma-Aldrich), 0.2 Units of *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (Sigma-Aldrich), and various concentrations of NADH or NADPH, to a volume of 90 μL. The reaction was initiated by the addition of 10 μL of [S]-acetolactate to a final concentration of 2.5 mM and a final volume of 100 μL. After 10 min incubations at 30° C., the reaction was quenched by withdrawing 50 μL of the reaction mixture and adding it to 150 μL of 0.1% formic acid. To measure the K_M of NADH and NADPH, the concentrations used were 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 and 1 mM.

To analyze for 2,3-dihydroxyisovalerate, 2 μL of the formic acid quenched reaction mixture was injected into a Waters Acquity HPLC equipped with Waters SOD mass spectrometer (Waters Corporation, Milford Mass.). The chromatography conditions were: flow rate (0.5 ml/min), on a Waters Acquity HSS T3 column (2.1 mm diameter, 100 mm length). Buffer A consisted of 0.1% (v/v) in water, Buffer B was 0.1% formic acid in acetonitrile. The sample was analyzed using 1% buffer B (in buffer A) for 1 min, followed by a linear gradient from 1% buffer B at 1 min to 75% buffer B at 1.5 min. The reaction product, 2,3-dihydroxyisovalerate, was detected by ionization at m/z=133, using electrospray ionization −30 V cone voltage. The amount of product 2,3-dihydroxyisovalerate was calculated by comparison to an authentic standard.

To calculate the K_M for NADH and NADPH, the rate data for DHIV formation measured in assays at a fixed concentration of S-acetolactate (2.5 mM) was fitted to the single substrate Michaelis-Menten equation, using a least-squares regression in Microsoft Excel, assuming saturating acetolactate concentration.

Construction of Plasmids pYZ058, pLH550, pLH556, and pLH702

pYZ058 (pHR81-P_{CUP1}-AlsS-P_{ILV5}-yeast KARI; SEQ ID NO: 176) was derived from pYZ090 (pHR81-P_{CUP1}-AlsS-P_{ILV5}-lactic KARI; SEQ ID NO: 195). pYZ090 was cut with PmeI and SfiI enzymes, and ligated with a PCR product of yeast KARI. The PCR product was amplified from genomic DNA of *Saccharomyces cerevisiae* BY4741 (Research Genetics Inc.) strain using upper primer 5'-catcatcacagtttaaacagtatgttgaagcaaatcaacttcggtgg-3' (SEQ ID NO: 272) and lower primer 5'-ggacgggccctgcaggccttattggttttctggtctcaactttctgac-3' (SEQ ID NO; 273), and digested with PmeI and SfiI enzymes. pYZ058 was confirmed by sequencing.

pLH550 (pHR81-PCUP1-AlsS-PILV5-Pf5.KARI, SEQ ID NO: 175) was derived from pYZ058 (SEQ ID NO: 176). The wild type Pf5.KARI gene was PCR amplified with OT1349 (5'-catcatcacagtttaaacagtatgaaagttttctacgataaagactgcgacc-3'; SEQ ID NO: 177) and OT1318 (5'-gcacttgataggcctgcagggccttagttcttggctttgtcgacgattttg-3'; SEQ ID NO: 178), digested with PmeI and SfiI enzymes and ligated with pYZ058 vector cut with PmeI and SfiI. The vector generated, pLH550, was confirmed by sequencing. pLH556 (SEQ ID NO: 138; FIG. **4**) was derived from pLH550 by digesting the vector with SpeI and NotI enzymes, and ligating with a linker annealed from OT1383 (5'-ctagtcaccggtggc-3', SEQ ID NO: 179) and OT1384 (5'-ggccgccaccggtga-3°, SEQ ID NO: 180) which contains overhang sequences for SpeI and NotI sites. This cloning step eliminates the AlsS gene and a large fragment of the PCUP1 promoter, with 160 bp residual upstream sequence that is not functional. pLH556 was confirmed by sequencing.

pHR81::ILV5p-K9D3 (pLH702, SEQ ID NO: 181) was derived from pLH556. The K9D3 mutant KARI gene was excised from vector pBAD-K9D3 using PmeI and SfiI enzymes, and ligated with pLH556 at PmeI and SfiI sites, replacing the Pf5.KARI gene with the K9D3 gene. The constructed vector was confirmed by sequencing.

Example 1

Construction of Yeast Isobutanol Pathway Strains Containing Various KARI Genes

To identify polypeptides having KARI activity and performance in yeast isobutanol production, biodiversity screening of KARI-encoding genes from various bacterial and fungal species was carried out. The KARI genes were codon optimized based on codon preferences of *Saccharomyces cerevisiae* genes where indicated in Table 10. For each KARI gene, a PmeI restriction site and additional 3 bp (AGT) was added to the 5' end with the sequence 5'-GTTTAAACAGT-3' (SEQ ID NO: 136) before the ATG start codon, and a SfiI restriction site was added to the 3' end with the sequence 5'-GGCCCTGCAGGCC-3' (SEQ ID NO: 137). All of the KARI genes were synthesized by GenScript USA Inc. (Piscataway, N.J.). Each KARI gene was subcloned into pHR81-P_{CUP1}-

AlsS-P_{Ilv5}-Pf5.Ilv5 vector (SEQ ID NO: 175) via the PmeI and SfiI sites (Ilv5 encodes for yeast ketol-acid reductoisomerase). This vector contains two expression cassettes: *Bacillus subtilis* acetolactate synthase (AlsS) gene under the yeast CUP1 promoter, and yeast Ilv5 gene controlled by the Ilv5 promoter. Sequence analysis was performed to confirm the KARI gene sequences.

The pHR81-PCUP1-AlsS-Pl_{IV5}-KARI vectors carrying the KARI genes were co-transformed with pLH468 (pRS423-P_{FBA1}-DHAD-P_{TDH3}-kivD-P_{GPM1}-hADH1; SEQ ID NO: 139) into host strain BP1135 (PNY1505: Example 8) (CEN.pk 113-7D delta ura3::loxP delta his3 delta pdc6 delta pdc1::ilvD.Sm delta pdc5::sadB delta gpd2::loxP delta fra2). The yeast transformants were selected on minimum drop-out media plates (SE-Ura-His, 2% ethanol) after 5-7 days at 30° C., and restreaked on SE-Ura-His to obtain cell patches after additional 3 day incubation. The cell patches were used for shake flask inoculation.

Example 2

Screening the KARI Diversity Collection for Isobutanol Production

The various KARI genes were evaluated based on their "effective productivities" in yeast. The effective productivity was determined after a certain period of growth under progressively oxygen-limited conditions (e.g. 48 h). The yeast biomass was calculated with the assumption that 1 OD_{600} of yeast cells is equivalent to 0.3 g/L.

The yeast isobutanol pathway strains carrying various KARI genes were inoculated into 10 mL SEG-Ura,His media with 0.2% glucose and 0.2% ethanol, and grown aerobically overnight at 30° C., to about 2 OD. The cultures were centrifuged and a portion of the cells were resuspended in SEG-Ura,His (2% glucose, 1% ethanol) to an initial OD_{600} of 0.4 in 25 mL total volume in a 125 mL shake flask. The shake flasks were dosed with a screw-on solid plastid cap, and the cultures were grown under progressively oxygen-limited conditions in the flask under minimal air and oxygen exchange with the outside environment. After 48 h incubation at 30° C., 250 RPM, the cultures were removed for OD_{600} measurement and HPLC analysis to measure isobutanol production.

From the KARI genes screened, as shown below, multiple had comparable or better isobutanol titers than *Lactococcus lactis* KARI. In particular, the K9 (*Anaerostipes caccae* DSM 14662) KARI clone showed a high isobutanol titer and effective isobutanol productivity, as measured after 48 h of growth under progressively oxygen-limited conditions (Table 10).

TABLE 10

Isobutanol titers and effective productivities from yeast isobutanol production strains carrying various KARI genes measured after 48 h of growth under progressively oxygen-limited conditions in shake flasks at 30° C.

KARI clone	SEQ ID NO: (nucleic acid, amino acid) *All nucleic acid seqs except LTS and S2 are codon-optimized	Isobutanol titer (g/L)	Effective Isobutanol Productivity (g/g)	Source Organism
B3K01 ("K1")	140, 141	2.6	4.1	*Bifidobacterium angulatum* DSM 20098
B3K02 ("K2")	142, 143	3.5	3.7	*Bifidobacterium dentium* ATCC 27678
B3K09 ("K9")	26, 27	4.3	5.2	*Anaerostipes caccae* DSM 14662
B3K25 ("K25")	375, 376	3.6	4.4	*Enterococcus gallinarum* EG2
B3K26 ("K26")	381, 382	4.4	3.2	*Streptococcus thermophilus* LMD-9
B3K29 ("K29")	377, 378	4.1	3.3	*Lactococcus lactis* subsp. *cremoris* MG1363
LTS	379, 380	2.7	3.1	*Lactococcus lactis*
B3K07 ("K7")	274, 275	3.7	2.8	*Clostridium beijerinckii* NCIMB 8052
S2	276, 277	3.6	1.5	*Zymomonas mobilis*

Example 3

KARI Enzyme Analysis of the Yeast Isobutanol Pathway Strains

IpOHA (N-isopropyl oxalylhydroxamic acid) is a mimic of a reaction intermediate for the reaction catalyzed by the KARI enzyme. It is a tight binding inhibitor that binds to the active site of the KARI enzyme. The synthesis of IpOHA and its tight binding to KARI from *E. coli* is described in literature (A. Aulabaugh and J. V. Schloss, Biochemistry, 1990, 29, 2824-2830). Its use for active site titration has not been reported before. IpOHA was synthesized from [^{14}C]-oxalate according to literature.

The yeast cultures from Example 2 were harvested and analyzed for KARI enzyme activities. 25 mL of the cultures was pelleted and resuspended in 10 mL of 50 mM Tris-HCl, pH 7.5. The cells were centrifuged again to remove the buffer and the cell pellets are stored at −70 C. The cell pellets were resuspended in 1 mL of 50 mM Tris-HCl pH 7.5 and sonicated. Soluble crude cell extracts were used to perform the enzyme assays. A portion of enzyme was incubated with a molar excess of [^{14}C]-IpOHA, and saturating concentrations of NAD(P)H and Mg^{2+}. Because a reversible, dilution-sensitive complex forms first, extract concentrations were kept high, to favor complexation and thus reduce the time taken for tight complex formation. Because it was not known a priori how long it would take each KARI to form the tight complex, two time points were taken for each sample to verify that the results agree. At the end of the incubation time, small molecules were separated from protein molecules by ultrafiltration using Microcon® (Millipore Inc., Billerica, Mass.), and the high molecular weight fraction was counted. The concentration of KARI in the sample in either μM or mg/ml was back-calculated from the ^{14}C dpm, the volumes, and the KARI subunit molecular weight. A fixed-time enzyme assay was run concurrently, and the data were used to calculate

U/ml. The specific activity was calculated by dividing U/ml by mg/ml for a given sample. The assumption made was that full activity and the ability to bind IpOHA are strictly correlated. The specific activities of the KARI enzymes thus measured are listed in Table 11. The KARI activity in "Units per mg" represents the activity per milligram of KARI enzyme as quantitated using the IpOHA assay. Total protein concentration was determined by the Bradford method, and the expression level of KARI is calculated by dividing KARI enzyme amount by the amount of total soluble cellular proteins.

TABLE 11

KARI enzyme activities measured by the IPOHA assay

KARI clone	KARI activity U/mg	KARI % total protein	Organism
B3K01	0.56	21	*Bifidobacterium angulatum* DSM 20098
B3K02	0.44	28	*Bifidobacterium dentium* ATCC 27678
B3K09	2.4	15	*Anaerostipes caccae* DSM 14662
B3K25	1.3	21	*Enterococcus gallinarum* EG2
B3K26	1.5	17	*Streptococcus thermophilus* LMD-9
B3K29	1.6	14	*Lactococcus lactis* subsp. *cremoris* MG1363
LTS	0.8	23.0	*Lactococcus lactis*

Example 4

Construction of a Site-Saturation Gene Library to Identify Variants Utilizing NADH with K_M Lower than Wild Type

To construct the pBAD-based bacterial expression vector for K9 KARI, the K9 KARI gene (synthesized by Genscript, Piscataway, N.J.) was subcloned into pBAD-ps-JEA1 vector (SEQ ID NO: 905) via the PmeI and SfiI sites. The ketol-acid reductolsomerase (KARI) from *Anaerostipes caccae* (called K9-KARI) was used for the library construction. One gene library was constructed using the commercially available kits, T4 polynucleotide kinase (PNK) (USB Corporation, Cleveland, Ohio, #70031Z) and Chang_IT Multiple Mutation Site Directed Mutagenesis Kit (USB Corporation, Cleveland, Ohio, #78480).

The oligonucleotides (K9_56_58_060210f: GAAGGANNKAAANNKTGGAAGAGAGC, SEQ ID NO: 144; and K9_56_58_060210r: GCTCTCTTCCAMNNTTTMNNTCCTTC, SEQ ID NO: 145) were synthesized by Integrated DNA Technologies, Inc (Coralville Iowa). They were first phosphorylated by T4 PNK. In brief, A 30 μl reaction mixture contained: 3.0 μl of 10× T4 PNK buffer supplied with the kit, 4.0 μl of primer (about 35 μM), 0.8 μl of 100 mM ATP mix, 0.6 μl T4 PNk and 22 μl of water. The reaction mixture was incubated at 37° C. for 1.0 hr and T4 PNK was then deactivated at 65° C. for 20 min.

The phosphorylated primers were then directly used for the subsequent PCR reaction to introduce the mutations at two sites into K9 KARI wild type using the kit. In brief, a 30 μl reaction mixture contained: 3.0 μl of 10× reaction buffer supplied with the kit, 3.0 μl of phosphorylated forward primer and reverse primer, 2.0 μl of K9 KARI wild type (50 ng/μl), 1.2 μl Chang_IT enzyme and 17.8 μl of water. This reaction mixture was placed into thin well 200 μl-capacity PCR tubes and the following PCR reaction program were used for the PCR: The starting temperature was 95° C. for 2 min followed by 30 heating/cooling cycles. Each cycle consisted of 95° C. for 30 sec, 55° C. for 30 sec, and 68° C. for 20 min. At the completion of the temperature cycling, the samples were kept at 68° C. for 25 min more, and then held at 4° C. for later processing. The PCR reaction was cleaned up using the Zymo DNA clean-up kit (Zymo Research Corporation, Orange Calif., #D4004). DNA was eluted out of membrane using 84 μl of water. The DNA template was removed with Dpn (Promega, Madison Wis., #R6231) at 37° C. for 3 hr (reaction mixture: 10 μl of 10× reaction buffer, 1.0 μl BSA, 6.0 μl of Dpn I and 83 μl cleaned PCR DNA). The Dpn I digested DNA was cleaned up again with Zymo DNA clean-up kit and digested again with Dpn I to completely remove the DNA template (reaction mixture: 1.5 μl of 10× reaction buffer, 0.15 μl BSA, 0.85 μl of Dpn I and 83 μl cleaned PCR DNA). The reaction mixture was directly used to transform an electrocompetent strain of *E. coli* Bw25113(ΔilvC) (described in U.S. Pat. No. 8,129,162, which is herein incorporated by reference in its entirety) using a BioRad Gene Pulser II (BioRad Laboratories Inc., Hercules, Calif.). The transformed clones were streaked on agar plates containing the LB medium and 100 μg/ml ampicillin (Cat#L1004, Teknova Inc, Hollister, Calif.) and incubated at 37° C. overnight. Clones were screened for activity using NADH. K_M for the variants was measured (Table 12).

TABLE 12

Kinetic Values for K9 KARI Variants in *E. coli* Extracts, as Determined via DHIV Formation Assays

Mutant	SEQ ID NO: (nucleic acid, amino acid)	Mutations	K_M (μM) (NADH)	K_M (μM) (NADPH)
K9 Wt	26, 27		326	0.2
AB1D1	28, 29	S56A	164	1
495B5	30, 31	S56A/S58H	44	4
AB1D3 (also referred to as "K9D3")	32, 33	S56A/S58D	38	9
AB1G9 (also referred to as "K9G9")	34, 35	S56AS58E	47	23

Example 5

Construction of Site Saturation Gene Libraries to Lower K_M for NADH

Based on work with *Pseudomonas fluorescens* KARI (PF5-KARI) positions 24, 33, 61, 80, 156 and 170 were targeted as mutagenesis targets for K9 KARI. Through multiple sequence alignment (MSA) between PF5-KARI and K9 KARI (FIG. **2**), the corresponding positions are 30, 39, 67, 86, 162, and 176.

To identify more mutagenesis targets, MSA of existing KARI enzymes (K1, K2, K7, K9, K25, K26, *L. Lactis* and S2), determined to produce isobutanol in a butanologen strain (see other examples) was used to identify more mutagenesis targets. Positions 41, 87, 131, 191, 227, and 246 were selected as mutagenesis targets.

The oligonucleotides targeting positions 30, 39, 41, 67, 86, 87, 131, 162, 176, 191, 227, and 246 were commercially synthesized by Integrated DNA Technologies, Inc (Coralville Iowa) (Table 13). Eight pairs of oligonucleotides targeting positions 30, 67, 131, 162, 176, 191, 227, and 246 were used to generate Megaprimers using Supermix from Invitrogen (Cat#10572-014, Invitrogen, Carlsbad, Calif.). For each PCR reaction, a pair of primers, any combination of one forward primer and one reverse primer encoding different positions

from those eight pairs of oligonucleotides (e.g. K9_30_101110f and K9_67_101110r), were used. There are total P_8^2 or 56 combinations. A 25 μl reaction mixture contained: 22.5 μl of Supermix solution, 1.0 μl of forward primer and 1.0 μl of reverse primer, 0.5 μl of AB1D3 DNA template (50 ng/μl). The mixture was placed in a thin well 200 μl tube for the PCR reaction in a Mastercycler gradient equipment (Brinkmann Instruments, Inc. Westbury, N.Y.). The following conditions were used for the PCR reaction: The starting temperature was 95° C. for 1.0 min followed by 35 heating/cooling cycles. Each cycle consisted of 95° C. for 20 sec, 55° C. for 20 sec, and 72° C. for 1.0 min. At the completion of the temperature cycling, the samples were kept at 72° C. for 2.0 min more, and then held awaiting sample recovery at 4° C. The PCR product was cleaned up using a DNA cleaning kit (Cat#D4003, Zymo Research, Orange, Calif.) as recommended by the manufacturer.

The Megaprimers were then used to generate gene libraries using the QuickChange II XL site directed mutagenesis kit (Catalog #200524, Stratagene, La Jolla Calif.). A 25 μl reaction mixture contained: 2.5 μl of 10× reaction buffer. 1.0 μl of 50 ng/μl template, 20.5 of Megaprimer, 0.5 of 40 mM dNTP mix, 0.5 μl pfu-ultra DNA polymerase. Except for the Megaprimer and the templates, all reagents used here were supplied with the kit indicated above. This reaction mixture was placed in a thin well 200 μl-capacity PCR tube and the following reactions were used for the PCR: The starting temperature was 95° C. for 30 sec followed by 25 heating/cooling cycles. Each cycle consisted of 95° C. for 30 sec, 55° C. for 1 min, and 68° C. for 6 min. At the completion of the temperature cycling, the samples were kept at 68° C. for 8 min more, and then held at 4° C. for later processing. The PCR reaction mixture was processed with Dpn I restriction enzyme same as that used in Example 4.

The oligonucleotides K9_37&39_101110f, K9_37&39_101110r and K9_86&87_101110f, K9_86&87_101110r were directly then used to generate gene libraries using the QuickChange II XL site directed mutagenesis kit. Two 25 d reaction mixtures for the two oligonucleotide sets, each 25 μl reaction mixture contained: 2.5 μl of 10× reaction buffer, 1.0 μl of 50 ng/μl template, 1.0 μl of forward primer, 1.0 μl reverse primer, 0.5 μl of 40 mM dNTP mix, 0.5 μl pfu-ultra DNA polymerase and 18.5 μl of water. The PCR program and the subsequent Dpn I processing are the same.

The Dpn I processed DNA mixture was cleaned up using Zymo DNA clean-up kit following the manufacturers protocol. The cleaned-up DNA was used to transform an electrocompetent strain of *E. coli* Bw25113 (ΔilvC) using a BioRad Gene Pulser II (Bio-Rad Laboratories Inc., Hercules, Calif.). The transformed clones were streaked on agar plates containing the LB medium and 100 μg/ml ampicillin (Cat#L1004, Teknova Inc. Hollister, Calif.) and incubated at 37° C. overnight. Clones were screened for improved activity using NADH. K_M for the improved mutants was measured (Table 14).

TABLE 13

Targeted position(s) of K9-KARI	Primers
30	K9_30_101110f: gactatcgccgttatcggtNNKggttctcaaggtcac SEQ ID NO: 146
	K9_30_101110r: GTGACCTTGAGAACCMNNACCGATAACGGCGATAGTC SEQ ID NO: 147
67	K9_67_101110f: gagctgaagaacaaggtNNKgaagtctacaccgctgc SEQ ID NO: 148
	K9_67_101110r: GCAGCGGTGTAGACTTCMNNACCTTGTTCTTCAGCTC SEQ ID NO: 149
131	K9_131_101110f: caaaggacgttgatgtcNNKatgatcgctccaaag SEQ ID NO: 150
	K9_131_101110r: CTTTGGAGCGATCATMNNGACATCAACGTCCTTT SEQ ID NO: 151
162	K9_162_101110f: gctgtcgaacaagacNNKactggcaaggctttg SEQ ID NO: 152
	K9_162_101110r: CAAAGCCTTGCCAGTMNNGTCTTGTTCGACAGC SEQ ID NO: 153
176	K9_176_101110f: gctttggcctacgctttaNNKatcggtggtgctagagc SEQ ID NO: 154
	K9_176_101110r: GCTCTAGCACCACCGATMNNTAAAGCGTAGGCCAAAGC SEQ ID NO: 155
191	K9_191_101110f: gaaactaccttcagaNNKgaaactgaaaccgac SEQ ID NO: 156
	K9_191_101110r: GTCGGTTTCAGTTTCMNNTCTGAAGGTAGTTTC SEQ ID NO: 157
227	K9_227_101110f: gccggttacgacccaNNKaacgcttacttcgaatg SEQ ID NO: 158
	K9_227_101110r: CATTCGAAGTAAGCGTTMNNTGGGTCGTAACCGGC SEO ID NO: 159
246	K9_246_101110f: gttgacttgatctacNNKtotggtttctccggtatgc SEQ ID NO: 160
	K9_246_101110r: GCATACCGGAGAAACCAGAMNNGTAGATCAAGTCAAC SEQ ID NO: 161
39, 41	K9_37&39_101110f: gttctcaaggtcacgctNNKgccNNKaatgctaaggaatcc SEQ ID NO: 162
	K9_37&39_101110r: GGATTCCTTAGCATTMNNGGCMNNAGCGTGACCTTGAGAAC SEQ ID NO: 163
86, 87	K9_86&87_101110f: gacatcattatgatcttgNNKNNKgatgaaaagcaggc SEQ ID NO: 164
	K9_86&87_101110r: GCCTGCTTTTCATCMNNMNNCAAGATCATAATGATGTC SEQ ID NO: 165

TABLE 14

List of some mutants with their measured K_M values

Mutant	SEQ ID NO: (nucleic acid, amino acid)	Mutations	K_M (μM) (NADH)	K_M (μM) (NADPH)
AO7A9	38, 39	S56A/S58D/I86T/N87P	15	7
AO7B5	36, 37	S56A/S58D/I86V/N87P	8	4
AO7H8	40, 41	S56A/S58D/N87P	8	6
AO7D8	42, 43	S56A/S58D/T131C/T191S	26	6
AO7F7	44, 45	S56A/S58D/T131V/T191A	28	7
AO7H7	46, 47	S56A/S58D/T191S	29	8

Example 6

Construction of a Combinatorial Library to Lower K_M or NADH

Based on the mutagenesis results (Example 4), T131L, T131A, T131V, T131M, T131C, T191D, T191C, T191S, and T191G are considered as beneficial mutations to improve K_M for NADH. A combinatorial library to introduce these beneficial mutations into AO7B5 was made.

All oligonucleotides were synthesized by the Integrated DNA Technologies, Inc (Coralville Iowa). They were first phosphorylated by T4 PNK. In brief, a 20 μl reaction mixture contained: 2.0 μl of 10× T4 PNK buffer supplied with the kit, 2.85 of primer (about 35 μM, 0.6 μl of 100 mM ATP mix, 0.4 μl T4 PNK and 14.15 μl of water. The reaction mixture was incubated at 37° C. for 1.0 hr and T4 PNK was then deactivated at 65° C. for 20 min.

The phosphorylated primers were then directly used for the subsequent PCR reaction to introduce the mutations at two sites into AO7B5 using the kit. In brief, a 50 reaction mixture contained: 5.0 μl of 10× reaction buffer supplied with the kit, 2.5 of phosphorylated forward primer (0.5 μl of each forward primer shown in Table 15), 2.5 μl reverse primer (0.625 of each forward primer shown at Table 15), 2.5 of A07B5 (50 ng/μl), 2.5 Chang_IT enzyme and 35 μl of water. This reaction mixture was placed into thin well 200 μl-capacity PCR tubes and the following PCR reaction program were used for the PCR: The starting temperature was 95° C. for 2 min followed by 30 heating/cooling cycles. Each cycle consisted of 95° C. for 30 sec, 55° C. for 30 sec, and 68° C. for 20 min. At the completion of the temperature cycling, the samples were kept at 68° C. for 25 min more, and then held at 4° C. for later processing. The PCR reaction was cleaned up using the Zymo DNA clean-up kit (Zymo Research Corporation, Orange Calif., #D4004). DNA was eluted out of membrane using 84 μl of water. The DNA template was removed with Dpn I (Promega, Madison Wis., #R6231) at 37° C. for 3 hr (reaction mixture: 10 μl of 10× reaction buffer, 1.0 μl BSA, 6.0 of Dpn I and 83 μl cleaned PCR DNA). The Dpn I digested DNA was cleaned up again with Zymo DNA clean-up kit and digested again with Dpn I to completely remove the DNA template (reaction mixture: 1.5 μl of 10× reaction buffer, 0.15 μl BSA, 0.85 μl of Dpn I and 83 μl cleaned PCR DNA).

The Dpn I processed DNA mixture was cleaned up using Zymo DNA clean-up kit following the manufacturer's protocol. The cleaned-up DNA was used to transform an electrocompetent strain of *E. coli* Bw25113 (ΔilvC) using a BioRad Gene Pulser II (Bio-Rad Laboratories Inc., Hercules, Calif.). The transformed clones were streaked on agar plates containing the LB medium and 100 μg/ml ampicillin (Cat#L1004, Teknova Inc. Hollister, Calif.) and incubated at 37° C. overnight. Clones were screened for improved activity using NADH, K_M for the improved mutants was measured (Table 16).

TABLE 15

Primers for example 6

Targeted position(s) of K9-KARI	Primers	
131	K9_131L_112210f:	ggacgttgatgtcTTGatgatcgctcc SEQ ID NO: 166
	K9_131A_112210f:	ggacgttgatgtcGCAatgatcgctcc SEQ ID NO: 167
	K9_131V_112210f:	ggacgttgatgtcGTTatgatcgctcc SEQ ID NO: 168
	K9_131M_112210f:	ggacgttgatgtcATGatgatcgctcc SEQ ID NO: 169
	K9_131C_112210f:	ggacgttgatgtcTGAatgatcgctcc SEQ ID NO: 170
191	K9_191D_112210r:	GGTTTCAGTTTCGTCTCTGAAGGTAGTTTC SEQ ID NO: 171
	K9_191C_112210r:	GGTTTCAGTTTCGCCTCTGAAGGTAGTTTC SEQ ID NO: 172
	K9_191S_112210r:	GGTTTCAGTTTCCGATCTGAAGGTAGTTTC SEQ ID NO: 173
	K9_191G_112210r:	GGTTTCAGTTTCGCCTCTGAAGGTAGTTTC SEQ ID NO: 174

TABLE 16

List of some mutants with their measured K_M values

Mutant	SEQ ID NO: (nucleic acid, amino acid)	Mutations	K_M (μM) (NADH)	K_M (μM) (NADPH)
AWB9	52, 53	S56A/S58D/I86V/N87P/T131A	10	4
AWC1	54, 55	S56A/S58D/I86V/N87P/T131V	9	3
AWD6	62, 63	S56A/S58D/I86V/N87P/T131V/T191S	5	2
AWD10	64, 65	S56A/S58D/I86V/N87P/T131A/T191C	8	3
AWF4	56, 57	S56A/S58D/I86V/N87P/N107S/T131V	7	3
AWF6	58, 59	S56A/S58D/I86V/N87P/T131V/T191D	7	2
AWG4	50, 51	S56A/S58D/I86V/N87P/T131M	7	3
AWH3	60, 61	S56A/S58D/I86V/N87P/T131V/T191G	6	2
AS6F1	48, 49	S56A/S58D/I86V/N87P/T131M/T191G	4	1

Example 7

Isobutanol Production from K9 KARI Variants

The following variants of K9 KARI were generated as described above.

TABLE 17

KARI variants and the corresponding yeast expression vectors

Clone	Yeast Vector	Nucleotide Point Mutation Locations					Amino Acid Mutation
Names	Name	166	168	172	173	174	Positions
WT K9 KARI	pHR81-PIlv5-KARI-K9	T	T	T	C	C	S56, S58
AB1G9	pHR81-PIlv5-KARI-K9.G9	G	G	G	A	G	S56A, S58E
495B5	pHR81-PIlv5-KARI-K9.B5	G	T	C	A	T	S56A, S58H
AB1D3	pHR81-PIlv5-KARI-K9.D3	G	T	G	A	T	S56A, S58D
AB1D1	pHR81-PIlv5-KARI-K9.D1	G	T	T	C	C	S56A

The yeast expression plasmids were made by subcloning of the variant KARI genes from *E. coli* vectors (pBAD.KARI) into pHR81-PIlv5-Pf5.KARI vector pLH556 (FIG. **4**, SEQ ID NO: 138) at PmeI and SfiI sites. Yeast pathway strains were made in PNY2204 host (MATa ura3Δ::loxP his3Δ pdc6Δ pdc1Δ::P[PDC1]-DHAD|ilvD_Sm-PDC1t-pUC19-loxP-kanMX-loxP-P[FBA1]-ALS|alsS_Bs-CYC1t pdc5Δ::P[PDC5]-ADH|sadB_Ax-PDC5t gpd2Δ::loxP fra2Δ adh1Δ::UAS(PGK1)P[FBA1]-kivD_Ll(y)-ADH1t; Example 13) by co-transforming the KARI vectors as pathway plasmid #1, and pBP915 (pRS423-P_{FBA1}-DHAD-P_{GPM1}-hADH1; SEQ ID NO: 182) as pathway plasmid #2. Transformants were patched to the same medium containing 2% glucose and 0.1% ethanol as carbon sources. Three patches were tested for isobutanol production under microaerobic conditions in serum vials. A done that was transformed with pBP915 and the pLH702 plasmid which expresses K9D3 was designated PNY1910.

Yeast colonies from the transformation on SE-Ura-His plates appeared after 5-7 days. The colonies were patched onto fresh SE-Ura-His plates, incubate at 30° C. for 3 days. The patched cells were inoculated into 25 mL SEG-Ura,His media with 0.2% glucose and 0.2% ethanol and grown aerobically for 1-2 days at 30° C., to 2-30D. The cells were centrifuged and re-suspended in 1 mL of SEG-Ura,His media (2% glucose, 0.1% ethanol, 10 mg/L ergosterol, 50 mM MES, pH 5.5, thiamine 30 mg/L, nicotinic acid 30 mg/L). A calculated amount of cells were transferred to 45 mL total volume of the same media for a starting OD=0.2 in a 60 mL serum vial, with the top closed tightly by a crimper. This step was done in the regular bio-hood in air. The serum vials were incubated at 300, 200 rpm for 2 days. At 48 h, the samples were removed for OD and HPLC analysis of glucose, isobutanol and pathway intermediates. 24 h samples were taken in an anaerobic chamber to maintain the anaerobic condition in the serum vials. In the initial phase of the 48 h incubation, the air present in the head space (~15 mL) and the liquid media is consumed by the growing yeast cells. After the oxygen in the head space is consumed, the culture becomes anaerobic. Therefore this experiment includes switching condition from aerobic to oxygen limiting and anaerobic conditions.

Of the four K9 variants, AB1 G9 and AB1D3 produced relatively high isobutanol titers, while 495B5 and AB1D1 have lower titer. Wild type K9 KARI strain produced the lowest titer. While not wishing to be bound by theory, it is believed that the lower titer is due to the shifted balance of NADH and NADPH when cells are switched from aerobic to anaerobic conditions. By this rationale, under anaerobic conditions, NADH concentration and availability increased significantly, favoring the variant KARI enzymes that use NADH. Based on the kinetic analysis, AB1G9 ("K9G9") and AB1D3 ("K9D3") mutants have relatively high K_M for NADPH (23 & 9.2 μM), in addition to their relative low K_M for NADH (47 & 38 μM). As comparison, 495B5 and AB1D1's K_M's are 2.5 and 1.1 μM respectively for NADPH, and wt K9's K_M is 0.10 μM. The low NADH K_M of AB1G9 and AB1D3, together with the high NADPH K_M of AB1 G9 and AB1D3 may have led to reduced NADPH utilization under anaerobic conditions, and relatively high NADH utilization. As evidence, AB1G9 and AB1D3 have lower glycerol accumulation (isobutanol:glycerol=3.3) compared to 495B5 and AB1D1 (2-3). The isobutanol:glycerol ratio is for the wild type K9 is 1:1 under the same switched aerobic to anaerobic condition.

TABLE 18

Kinetic properties of wild type and variant K9 KARI enzymes, and isobutanol titer and productivity measured from aerobic to anaerobic switch experiment in serum vials.

Clone Names	NADPH K_M (NADPH)	NADPH V_{max} (U/mg)	NADH K_M (NADH)	NADH V_{max} (U/mg)	Isobutanol g/L	Effective Isobutanol Productivity (g/g cells)
WT K9 KARI	0.19	2.0	326	1.5	0.9	3.1
AB1G9	23	2.4	47	2.0	3.4	10.5
495B5	3.5	2.5	44	1.9	2.2	9.2
AB1D3	9.2	2.1	38	1.9	3.3	10.8
AB1D1	1.1	2.8	164	2.1	2.3	9.5

Example 8

Construction of *Saccharomyces cerevisiae* Strains BP1135 (PNY1505) and PNY1507 and Isobutanol-Producing Derivatives

This example describes construction of *Saccharomyces cerevisiae* strains BP1135 and PNY1507. These strains were derived from PNY1503 (BP1064). PNY1503 was derived from CEN.PK 113-7D (CBS 8340; Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversiry Centre, Netherlands). BP1135 contains an additional deletion of the FRA2

gene. PNY1507 was derived from BP1135 with additional deletion of the ADH1 gene, with integration of the kivD gene from *Lactococcus lactis*, codon optimized for expression in *Saccharomyces cerevisiae*, into the ADH1 locus.

Deletions/integrations were created by homologous recombination with PCR fragments containing regions of homology upstream and downstream of the target gene and the URA3 gene for selection of transformants. The URA3 gene was removed by homologous recombination to create a scarless deletion/integration.

The scarless deletion/integration procedure was adapted from Akada et al., *Yeast,* 23:399, 2006. In general, the PCR cassette for each deletion/integration was made by combining four fragments, A-B-U-C, and the gene to be integrated by cloning the individual fragments into a plasmid prior to the entire cassette being amplified by PCR for the deletion/integration procedure. The gene to be integrated was included in the cassette between fragments A and B. The PCR cassette contained a selectable/counter-selectable marker, URA3 (Fragment U), consisting of the native CEN.PK 113-7D URA3 gene, along with the promoter (250 bp upstream of the URA3 gene) and terminator (150 bp downstream of the URA3 gene) regions. Fragments A and C (each approximately 100 to 500 bp long) corresponded to the sequence immediately upstream of the target region (Fragment A) and the 3° sequence of the target region (Fragment C), Fragments A and C were used for integration of the cassette into the chromosome by homologous recombination. Fragment B (500 bp long) corresponded to the 500 bp immediately downstream of the target region and was used for excision of the URA3 marker and Fragment C from the chromosome by homologous recombination, as a direct repeat of the sequence corresponding to Fragment B was created upon integration of the cassette into the chromosome.

FRA2 Deletion

The FRA2 deletion was designed to delete 250 nucleotides from the 3' end of the coding sequence, leaving the first 113 nucleotides of the FRA2 coding sequence intact. An in-frame stop codon was present 7 nucleotides downstream of the deletion. The four fragments for the PCR cassette for the starless FRA2 deletion were amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs; Ipswich, Mass.) and CEN.PK 113-7D genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen; Valencia, Calif.). FRA2 Fragment A was amplified with primer oBP594 (SEQ ID NO: 183) and primer oBP595 (SEQ ID NO: 184), containing a 5' tail with homology to the 5' end of FRA2 Fragment B. FRA2 Fragment B was amplified with primer oBP596 (SEQ ID NO: 185), containing a 5' tail with homology to the 3' end of FRA2 Fragment A, and primer oBP597 (SEQ ID NO:186), containing a 5' tail with homology to the 5' end of FRA2 Fragment U. FRA2 Fragment U was amplified with primer oBP598 (SEQ ID NO: 187), containing a 5' tail with homology to the 3' end of FRA2 Fragment B, and primer oBP599 (SEQ ID NO: 188), containing a 5' rail with homology to the 5' end of FRA2 Fragment C. FRA2 Fragment C was amplified with primer oBP600 (SEQ ID NO:189), containing a 5' tail with homology to the 3' end of FRA2 Fragment U, and primer oBP601 (SEQ ID NO:190). PCR products were purified with a PCR Purification kit (Qiagen). FRA2 Fragment AB was created by overlapping PCR by mixing FRA2 Fragment A and FRA2 Fragment B and amplifying with primers oBP594 (SEQ ID NO:183) and oBP597 (SEQ ID NO:186). FRA2 Fragment UC was created by overlapping PCR by mixing FRA2 Fragment U and FRA2 Fragment C and amplifying with primers oBP598 (SEQ ID NO:187) and oBP601 (SEQ ID NO:190). The resulting PCR products were purified on an agarose gel followed by a Gel Extraction kit (Qiagen). The FRA2 ABUC cassette was created by overlapping PCR by mixing FRA2 Fragment AB and FRA2 Fragment UC and amplifying with primers oBP594 (SEQ ID NO:183 and oBP601 (SEQ ID NO:190). The PCR product was purified with a PCR Purification kit (Qiagen).

Competent cells of PNY1503 were made and transformed with the FRA2 ABUC PCR cassette using a Frozen-EZ Yeast Transformation II kit (Zymo Research; Orange, Calif.). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 1% ethanol at 30° C. Transformants with a fra2 knockout were screened for by PCR with primers oBP602 (SEQ ID NO:191) and oBP603 (SEC) ID NO:192) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). A correct transformant was grown in YPE (yeast extract, peptone, 1% ethanol) and plated on synthetic complete medium containing 5-fluoro-orotic acid (0.1%) at 30° C. to select for isolates that lost the URA3 marker. The deletion and marker removal were confirmed by PCR with primers oBP602 (SEQ ID NO:191) and oBP603 (SEQ ID NO:192) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The absence of the FRA2 gene from the isolate was demonstrated by a negative PCR result using primers specific for the deleted coding sequence of FRA2, oBP605 (SEQ ID NO:193) and oBP606 (SEQ ID NO:194). The correct isolate was selected as strain CEN.PK 113-7D MATa ura3Δ::loxP his3Δ pdc6Δ pdc1Δ::P[PDC1]-DHAD|ilvD_Sm-PDC1t pdc5Δ::P [PDC5]-ADH|sadB_Ax-PDC5t gpd2Δ::loxP fra2Δ and designated as PNY1505 (BP1135).

This strain was transformed with isobutanol pathway plasmids (pYZ090, SEQ ID NO: 195) and pLH468 (SEQ ID NO: 139), and one clone was designated BPI 168 (PNY1506).

pYZ090 (SEQ ID NO: 195) was constructed to contain a chimeric gene having the coding region of the alsS gene from *Bacillus subtilis* (nt position 457-2172) expressed from the yeast CUP1 promoter (nt 2-449) and followed by the CYC1 terminator (nt 2181-2430) for expression of ALS, and a chimeric gene having the coding region of the ilvC gene from *Lactococcus lactis* (nt 3634-4656) expressed from the yeast ILV5 promoter (2433-3626) and followed by the ILV5 terminator (nt 4682-5304) for expression of KARI.

ADH1 Deletion and kivD_Ll(y) Integration

The ADH1 gene was deleted and replaced with the kivD coding region from *Lactococcus lactis* codon optimized for expression in *Saccharomyces cerevisiae*. The scarless cassette for the ADH1 deletion-kivD_Ll(y) integration was first cloned into plasmid pUC19-URA3MCS, as described in U.S. Apple. No. 61/356,379, filed Jun. 18, 2010, incorporated herein by reference. The vector is pUC19 based and contains the sequence of the URA3 gene from *Saccharomyces cerevisiae* CEN.PK 113-7D situated within a multiple cloning site (MCS), pUC19 contains the pMB1 replicon and a gene coding for beta-lactamase for replication and selection in *Escherichia coli*. In addition to the coding sequence for URA3, the sequences from upstream (250 bp) and downstream (150 bp) of this gene are present for expression of the URA3 gene in yeast. The vector can be used for cloning purposes and can be used as a yeast integration vector.

The kivD coding region from *Lactococcus* lactic codon optimized for expression in *Saccharomyces cerevisiae* was amplified using pLH468 (SEQ ID NO:139) as template with primer oBP562 (SEQ ID NO:197), containing a PmeI restriction site, and primer oBP563 (SEQ ID NO:198), containing a 5' tail with homology to the 5' end of ADH1 Fragment B. ADH1 Fragment B was amplified from genomic DNA prepared as above with primer oBP564 (SEQ ID NO:199), con-

taining a 5' tail with homology to the 3' end of kivD_Ll(y), and primer oBP565 (SEQ ID NO:200), containing a FseI restriction site. PCR products were purified with a PCR Purification kit (Qiagen). kivD_Ll(y)-ADH1 Fragment B was created by overlapping PCR by mixing the kivD_Ll(y) and ADH1 Fragment B PCR products and amplifying with primers oBP562 (SEQ ID NO:197) and oBP565 (SEQ ID NO:200). The resulting PCR product was digested with PmeI and FseI and ligated with T4 DNA ligase into the corresponding sites of pUC19-URA3MCS after digestion with the appropriate enzymes. ADH1 Fragment A was amplified from genomic DNA with primer oBP505 (SEQ ID NO:201), containing a SacI restriction site, and primer oBP506 (SEQ ID NO:202), containing an AscI restriction site. The ADH1 Fragment A PCR product was digested with SacI and AscI and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing kivD_Ll(y)-ADH1 Fragment B. ADH1 Fragment C was amplified from genomic DNA with primer oBP507 (SEQ ID NO:203), containing a Pad restriction site, and primer oBP508 (SEQ ID NO:204), containing a SalI restriction site. The ADH1 Fragment C PCR product was digested with Pad and SalI and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing ADH1 Fragment A-kivD_Ll(y)-ADH1 Fragment B. The hybrid promoter UAS(PGK1)-P_{FBA1} was amplified from vector pRS316-UAS(PGK1)-P_{FBA1}-GUS (SEQ ID NO:209) with primer oBP674 (SEQ ID NO:205), containing an AscI restriction site, and primer oBP675 (SEQ ID NO:206), containing a PmeI restriction site. The UAS(PGK1)-P_{FBA1} PCR product was digested with AscI and PmeI and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing kivD_Ll(y)-ADH1 Fragments ABC. The entire integration cassette was amplified from the resulting plasmid with primers oBP505 (SEQ ID NO:201) and oBP508 (SEQ ID NO:204) and purified with a PCR Purification kit (Qiagen).

Competent cells of PNY1505 were made and transformed with the ADH1-kivD_Ll(y) PCR cassette constructed above using a Frozen-EZ Yeast Transformation H kit (Zymo Research). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 1% ethanol at 30° C. Transformants were grown in YPE (1% ethanol) and plated on synthetic complete medium containing 5-fluoroorotic acid (0.1%) at 30° C. to select for isolates that lost the URA3 marker. The deletion of ADH1 and integration of kivD_Ll(y) were confirmed by PCR with external primers oBP495 (SEQ ID NO:207) and oBP496 (SEQ ID NO:208) and with kivD_Ll(y) specific primer oBP562 (SEQ ID NO:197) and external primer oBP496 (SEQ ID NO:208) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The correct isolate was selected as strain CEN.PK 113-7D MATa ura3Δ::loxP his3Δ pdc6Δ pdc1Δ::P[PDC1]-DHAD|ilvD_.Sm-PDC1tpdc5Δ::P[PDC5]-ADH|sadB_Ax-PDC5t gpd2Δ::loxP fra2Δ adh1Δ::UAS(PGK1)P[FBA1]-kivD_Ll(y)-ADH1t and designated as PNY1507 (BP1201). PNY1507 was transformed with isobutanol pathway plasmids pYZ090 (SEQ ID NO:195) and pBP915 (SEQ ID NO: 182) and the resultant strain was named PNY1513.

Construction of the pRS316-UAS(PGK1)-FBA1p-GUS Vector

To clone a cassette UAS(PGK1)-FBA1p (SEQ ID NO:766, first a 602 bp FBA1 promoter (FBA1p) was PCR-amplified from genomic DNA of CEN.PK with primers T-FBA1(SalI) (SEQ ID NO:767) and B-FBA1(SpeI) (SEQ ID NO:768), and cloned into SalI and SpeI sites on the plasmid pWS358-PGK1p-GUS (SEQ ID NO:769) after the PGK1p promoter was removed with a SalI/SpeI digest of the plasmid, yielding pWS358-FBA1p-GUS. The pWS358-PGK1p-GUS plasmid was generated by inserting a PGK1p and beta-glucuronidase gene (GUS) DNA fragments into multiple cloning site of pWS358, which was derived from pRS423 vector (Christianson et al., Gene, 110:119-122, 1992). Secondly, the resulting pWS358-FBA1p-GUS plasmid was digested with SalI and SacI, a DNA fragment containing a FBA1p promoter, GUS gene, and FBAt terminator gel-purified, and cloned into SalI/SacI sites on pRS316 to create pRS316-FBA1p-GUS. Thirdly, a 118 bp DNA fragment containing an upstream activation sequence (UAS) located between positions −519 and −402 upstream of the 3-phosphoglycerate kinase (PGK1) open reading frame, namely UAS(PGK1), was PCR-amplified from genomic DNA of CEN.PK with primers T-U/PGK1 (KpnI) (SEQ ID NO:770) and B-U/PGK1(SalI) (SEQ ID NO:771). The PCR product was digested with KpnI and SalI and cloned into KpnI/SalI sites on pRS316-FBA1p-GUS to create pRS316-UAS(PGK1)-FBA1p-GUS.

Example 9

Improved Recombinant Host Cells Comprising Elimination of ALD6

The purpose of this example is to describe methods to modify a yeast host strain for improved production of isobutanol. These modifications include integration of genes encoding isobutyraldehyde reductase activity and elimination of the native genes ALD6 and YMR226C, encoding NADP+-dependent acetaldehyde dehydrogenase and a NADPH-dependent dehydrogenase, respectively.

Construction of *S. cerevisiae* Strain PNY2211

PNY2211 was constructed in several steps from *S. cerevisiae* strain PNY1507 (Example 8) as described in the following paragraphs. First PNY1507 was modified to contain a phosophoketolase gene. Next, an acetolactate synthase gene (alsS) was added to the strain, using an integration vector targeted to sequences adjacent to the phosphokeloase gene. Finally, homologous recombination was used to remove the phosphoketolase gene and integration vector sequences, resulting in a starless insertion of alsS in the intergenic region between pdc1Δ::ilvD (described in Example 12) and the native TRX1 gene of chromosome XII. The resulting genotype of PNY2211 is MATa ura3Δ:loxP his3Δ pdc6Δ pdc1Δ::P[PDC1]-DHAD|ilvD_.Sm-PDC1t-P[FBA1]-ALS|alsS_Bs-CYC1t pdc5Δ::P[PDC5]-ADH| sadB_Ax-PDC5t gpd2Δ:loxP fra2Δ adh1Δ::UAS(PGK1)P[FBA1]-kivD_Ll(y)-ADH1t.

A phosphoketolase gene cassette was introduced into PNY1507 by homologous recombination. The integration construct was generated as follows. The plasmid pRS423::CUP1-alsS+FBA-budA (previously described in US2009/0305363, which is herein incorporated by reference in its entirety) was digested with NotI and XmaI to remove the 1.8 kb FBA-budA sequence, and the vector was religated after treatment with Klenow fragment. Next, the CUP1 promoter was replaced with a TEF1 promoter variant (M4 variant previously described by Nevoigt et al. *Appl. Environ, Microbiol.* 72: 5266-5273 (2006), which is herein incorporated by reference in its entirety) via DNA synthesis and vector construction service from DNA2.0 (Menlo Park, Calif.). The resulting plasmid, pRS423::TEF(M4)-alsS was cut with StuI and MluI (removes 1.6 kb portion containing part of the alsS gene and CYC1 terminator), combined with the 4 kb PCR product generated from pRS426::GPD-xpk1+ADH-eutD (SEQ ID NO:383) with primers N1176 (SEC) ID NO:282) and N1177 (SEQ ID NO:283) and an 0.8 kb PCR product DNA (SEQ ID

NO: 284) generated from yeast genomic DNA (ENO1 promoter region) with primers N822 (SEQ ID NO:285) and N1178 (SEQ ID NO:286) and transformed into *S. cerevisiae* strain BY4741 (ATCC #201388); gap repair cloning methodology, see Ma et al. *Gene* 58:201-216 (1987). Transformants were obtained by plating cells on synthetic complete medium without histidine. Proper assembly of the expected plasmid (pRS423::TEF(M4)-xpk1+ENO1-eutD, SEQ ID NO:293) was confirmed by PCR (primers N821 (SEQ ID NO:287) and N1115 (SEQ ID NO:288)) and by restriction digest (BgII). Two clones were subsequently sequenced. The 3.1 kb TEF (M4)-xpk1 gene was isolated by digestion with SacI and NotI and cloned into the pUC19-URA3::ilvD-TRX1 vector (Clone A, cut with AflII). Cloning fragments were treated with Klenow fragment to generate blunt ends for ligation. Ligation reactions were transformed into *E. coli* Stb13 cells, selecting for ampicillin resistance. Insertion of TEF(M4)-xpk1 was confirmed by PCR (primers N1110 (SEQ ID NO:367) and N1114 (SEQ ID NO:290)). The vector was linearized with AflII and treated with Klenow fragment. The 1.8 kb KpnI-HincII geneticin resistance cassette (SEQ ID NO: 384) was cloned by ligation after Klenow fragment treatment. Ligation reactions were transformed into *E. coli* Stb13 cells, selecting for ampicillin resistance. Insertion of the geneticin cassette was confirmed by PCR (primers N160SeqF5 (SEQ ID NO:210) and BK468 (SEQ ID NO:368)). The plasmid sequence is provided as SEQ ID NO: 291 (pUC19-URA3::pdc1::TEF(M4)-xpk1::kan).

The resulting integration cassette (pdc1::TEF(M4)-xpk1::KanMX::TRX1) was isolated (AscI and NaeI digestion generated a 5.3 kb band that was gel purified) and transformed into PNY1507 using the Zymo Research Frozen-EZ Yeast Transformation Kit (Cat. No. T2001). Transformants were selected by plating on YPE plus 50 μg/ml G418. Integration at the expected locus was confirmed by PCR (primers N886 (SEQ ID NO:211) and N1214 (SEQ ID NO:281)). Next, plasmid pRS423::GAL1p-Cre (SEQ ID NO:271), encoding Cre recombinase, was used to remove the loxP-flanked KanMX cassette. Proper removal of the cassette was confirmed by PCR (primers oBP512 (SEQ ID NO: 337) and N160SeqF5 (SEQ ID NO:210)). Finally, the alsS integration plasmid described in Example 13, pUC19-kan::pdc1::FBA-alsS::TRX1, clone A) was transformed into this strain using the included geneticin selection marker. Two integrants were tested for acetolactate synthase activity by transformation with plasmids pYZ090ΔalsS (SEQ ID NO:371) and pBP915 (SEQ ID NO:182) (transformed using Protocol #2 in Amberg, Burke and Strathern "Methods in Yeast Genetics" (2005)), and evaluation of growth and isobutanol production in glucose-containing media (methods for growth and isobutanol measurement are as follows: All strains were grown in synthetic complete medium, minus histidine and uracil containing 0.3% glucose and 0.3% ethanol as carbon sources (10 mL medium in 125 mL vented Erlenmeyer flasks (VWR Cat. No. 89095-260). After overnight incubation (30° C., 250 rpm in an Innova®40 New Brunswick Scientific Shaker), cultures were diluted back to 0.2 OD (Eppendorf BioPhotometer measurement) in synthetic complete medium containing 2% glucose and 0.05% ethanol (20 ml medium in 125 mL tightly-capped Erlenmeyer flasks (VWR Cat. No. 89095-260)). After 48 hours incubation (30° C., 250 rpm in an Innova®40 New Brunswick Scientific Shaker), culture supernatants (collected using Spin-X centrifuge tube filter units, Costar Cat. No. 8169) were analyzed by HPLC per methods described in U.S. Appl. Pub. No. 2007/0092957, which is herein incorporated by reference in its entirety) One of the two clones was positive and was named PNY2218.

PNY2218 was treated with Cre recombinase and the resulting clones were screened for loss of the xpk1 gene and pUC19 integration vector sequences by PCR (primers N886 (SEQ ID NO: 211) and N160SeqR5 (SEQ ID NO: 388)). This left only the alsS gene integrated in the pdc1-TRX1 intergenic region after recombination the DNA upstream of xpk1 and the homologous DNA introduced during insertion of the integration vector (a "scarless" insertion since vector, marker gene and loxP sequences are lost). Although this recombination could have occurred at any point, the vector integration appeared to be stable even without geneticin selection and the recombination event was only observed after introduction of the Cre recombinase. One clone was designated PNY2211.

An isolate of PNY2218 containing the plasmids pYZ090ΔalsS and pBP915 was designated PNY2209.

PNY1528 (hADH Integrations in PNY2211)

Deletions/integrations were created by homologous recombination with PCR products containing regions of homology upstream and downstream of the target region and the URA3 gene for selection of transformants. The URA3 gene was removed by homologous recombination to create a scarless deletion/integration.

YPRCΔ15 Deletion and Horse Liver adh Integration

The YPRCΔ15 locus was deleted and replaced with the horse liver adh gene, codon optimized for expression in *Saccharomyces cerevisiae*, along with the PDC5 promoter region (538 bp) from *Saccharomyces cerevisiae* and the ADH1 terminator region (316 bp) from *Saccharomyces cerevisiae*. The scarless cassette for the YPRCΔ15 deletion-P[PDC5]-adh_HL(y)-ADH1t integration was first cloned into plasmid pUC19-URA3MCS (described in Example 8).

Fragments A-B-U-C were amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs; Ipswich, Mass.) and CEN.PK 113-7D genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen; Valencia, Calif.). YPRCΔ15 Fragment A was amplified from genomic DNA with primer oBP622 (SEQ ID NO: 212), containing a KpnI restriction site, and primer oBP623 (SEQ ID NO: 213), containing a 5' tail with homology to the 5' end of YPRCΔ15 Fragment B. YPRCΔ15 Fragment B was amplified from genomic DNA with primer oBP624 (SEQ ID NO: 214), containing a 5° tail with homology to the 3' end of YPRCΔ15 Fragment A, and primer oBP625 (SEQ ID NO: 215), containing a FseI restriction site. PCR products were purified with a PCR Purification kit (Qiagen). YPRCΔ15 Fragment A—YPRCΔ15 Fragment B was created by overlapping PCR by mixing the YPRCΔ15 Fragment A and YPRCΔ15 Fragment B PCR products and amplifying with primers oBP622 (SEQ ID NO: 212) and oBP625 (SEQ ID NO: 215). The resulting PCR product was digested with KpnI and FseI and ligated with T4 DNA ligase into the corresponding sites of pUC19-URA3MCS after digestion with the appropriate enzymes. YPRCΔ15 Fragment C was amplified from genomic DNA with primer oBP626 (SEQ ID NO: 216), containing a Not restriction site, and primer oBP627 (SEQ ID NO: 217), containing a PacI restriction site. The YPRCΔ15 Fragment C PCR product was digested with NotI and PacI and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing YPRCΔ15 Fragments AB. The PDC5 promoter region was amplified from CEN.PK 113-7D genomic DNA with primer HY21 (SEQ ID NO: 218), containing an AscI restriction site, and primer HY24 (SEQ ID NO: 219), containing a 5° tail with homology to the 5' end of adh_Hl(y). adh_Hl(y)-ADH1t was amplified from pBP915 (SEQ ID NO: 182) with primers HY25 (SEQ ID NO: 220), containing a 5' tail with homology to the 3' end of P[PDC5], and HY4 (SEQ ID NO: 221), containing a PmeI restriction

site. PCR products were purified with a PCR Purification kit (Qiagen). P[PDC5]-adh_HL(y)-ADH1t was created by overlapping PCR by mixing the P[PDC5] and adh_HL(y)-ADH1t PCR products and amplifying with primers HY21 (SEQ ID NO: 218) and HY4 (SEQ ID NO: 221). The resulting PCR product was digested with AscI and PmeI and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing YPRCΔ15 Fragments ABC. The entire integration cassette was amplified from the resulting plasmid with primers oBP622 (SEQ ID NO: 212) and oBP627 (SEQ ID NO: 217).

Competent cells of PNY2211 were made and transformed with the YPRCΔ15 deletion-P[PDC5]-adh_HL(y)-ADH1t integration cassette PCR product using a Frozen-EZ Yeast Transformation II kit (Zymo Research; Orange, Calif.). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 1% ethanol at 30° C., Transformants were screened for by PCR with primers URA3-end F (SEQ ID NO: 222) and oBP637 (SEQ ID NO: 224). Correct transformants were grown in YPE (1% ethanol) and plated on synthetic complete medium supplemented with 1% EtOH and containing 5-fluoro-orotic acid (0.1%) at 30 C to select for isolates that lost the URA3 marker. The deletion of YPRCΔ15 and integration of P[PDC5]-adh_HL(y)-ADH1t were confirmed by PCR with external primers oBP636 (SEQ ID NO: 223) and oBP637 (SEQ ID NO: 224) using genomic DNA prepared with a YeaStar Genomic DNA kit (Zymo Research). A correct isolate of the following genotype was selected for further modification: CEN.PK 113-7D MATa ura3Δ::loxP his3Δ pdc6Δ pdc1Δ::P[PDC1]-DHAD|ilvD_Sm-PDC1t-P[FBA1]-ALS|alsS_Bs-CYC1t pdc5Δ::P[PDC5]-ADH|sadB_.Ax-PDC5t gpd2Δ::loxP fra2Δ adh1Δ::UAS(PGK1)P[FBA1]-kivD_Ll(y)-ADH1t yprcΔ 15Δ::P[PDC5]-ADH|adh_Hl-ADH1 t.

Horse Liver adh Integration at fra2Δ

The horse liver adh gene, codon optimized for expression in *Saccharomyces cerevisiae*, along with the PDC1 promoter region (870 bp) from *Saccharomyces cerevisiae* and the ADH1 terminator region (316 bp) from *Saccharomyces cerevisiae*, was integrated into the site of the fra2 deletion. The scarless cassette for the fra2Δ-P[PDC1]-adh_HL(y)-ADH1t integration was first cloned into plasmid pUC19-URA3MCS.

Fragments A-B-U-C were amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs; Ipswich, Mass.) and CEN.PK 113-7D genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen; Valencia, Calif.). fra2Δ Fragment C was amplified from genomic DNA with primer oBP695 (SEQ ID NO: 229), containing a NotI restriction site, and primer oBP696 (SEQ ID NO: 230), containing a Pad restriction site. The fra2Δ Fragment C PCR product was digested with NotI and Pad and ligated with T4 DNA ligase into the corresponding sites of pUC19-URA3MCS. fra2Δ Fragment B was amplified from genomic DNA with primer oBP693 (SEQ ID NO: 227), containing a PmeI restriction site, and primer oBP694 (SEQ ID NO: 228), containing a FseI restriction site. The resulting PCR product was digested with PmeI and FseI and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing fra2Δ fragment C after digestion with the appropriate enzymes. fra2Δ Fragment A was amplified from genomic DNA with primer oBP691 (SEQ ID NO: 225), containing BamHI and AsiSI restriction sites, and primer oBP692 (SEQ ID NO: 226), containing AscI and SwaI restriction sites. The fra2Δ fragment A PCR product was digested with BamHI and AscI and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing fra2Δ fragments BC after digestion with the appropriate enzymes. The PDC1 promoter region was amplified from CEN.PK 113-7D genomic DNA with primer HY16 (SEQ ID NO: 231), containing an AscI restriction site, and primer HY19 (SEQ ID NO: 232), containing a 5 tail with homology to the 5' end of adh_Hl(y). adh_Hl(y)-ADH1t was amplified from pBP915 with primers HY20 (SEQ ID NO: 233), containing a 5' tail with homology to the 3' end of P[PDC1], and HY4 (SEQ ID NO: 221), containing a PmeI restriction site. PCR products were purified with a PCR Purification kit (Qiagen). P[PDC1]-adh_HL(y)-ADH1t was created by overlapping PCR by mixing the P[PDC1] and adh_HL(y)-ADH1t PCR products and amplifying with primers HY16 (SEQ ID NO: 231) and HY4 (SEQ ID NO: 221). The resulting PCR product was digested with AscI and PmeI and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing fra2Δ Fragments ABC. The entire integration cassette was amplified from the resulting plasmid with primers oBP691 (SEQ ID NO: 225) and oBP696 (SEQ ID NO: 230).

Competent cells of the PNY2211 variant with adh_Hl(y) integrated at YPRCΔ15 were made and transformed with the fra2Δ-P[PDC1]-adh_HL(y)-ADH1t integration cassette PCR product using a Frozen-EZ Yeast Transformation II kit (Zymo Research). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 1% ethanol at 30° C. Transformants were screened for by PCR with primers URA3-end F (SEQ ID NO: 222) and oBP731 (SEQ ID NO: 235). Correct transformants were grown in YPE (1% ethanol) and plated on synthetic complete medium supplemented with 1% EtOH and containing 5-fluoro-orotic acid (0.1%) at 30° C. to select for isolates that lost the URA3 marker. The integration of P[PDC1]-adh_HL(y)-ADH1t was confirmed by colony PCR with internal primer HY31 (SEQ ID NO: 236) and external primer oBP731 (SEQ ID NO: 235) and PCR with external primers oBP730 (SEQ ID NO: 234) and oBP731 (SEQ ID NO: 235) using genomic DNA prepared with a YeaStar Genomic DNA kit (Zymo Research). A correct isolate of the following genotype was designated PNY1528: CEN.PK 113-7D MATa ura3Δ::loxP his3Δ pdc6Δ pdc1Δ::P[PDC1]-DHAD|ilvD_Sm-PDC1t-P[FBA1]-ALS|alsS_Bs-CYC1t pdc5Δ::P[PDC5]-ADH|sadB_Ax-PDC5t gpd2Δ::loxP fra2Δ::P[PDC1]-ADH|adh_Hl-ADH1t adh1Δ::UAS(PGK1)P[FBA1]-kivD_Ll(y)-ADH1t yprcΔ15Δ::P[PDC5]-ADH|adh_Hl-ADH1t.

PNY2237 (Scarless YMR226C Deletion)

The gene YMR226C was deleted from *S. cerevisiae* strain PNY1528 by homologous recombination using a PCR amplified 2.0 kb linear scarless deletion cassette. The cassette was constructed from spliced PCR amplified fragments comprised of the URA3 gene, along with its native promoter and terminator as a selectable marker, upstream and downstream homology sequences flanking the YMR226C gene chromosomal locus to promote integration of the deletion cassette and removal of the native intervening sequence and a repeat sequence to promote recombination and removal of the URA3 marker. Forward and reverse PCR primers (N1251 and N1252, SEQ ID NOs: 247 and 248, respectively), amplified a 1,208 bp URA3 expression cassette originating from pLA33 (pUC19::loxP-URA3-loxP (SEQ ID NO: 268)). Forward and reverse primers (N1253 and N1254, SEQ ID NOs: 249 and 250, respectively), amplified a 250 bp downstream homology sequence with a 3° URA3 overlap sequence tag from a genomic DNA preparation of *S. cerevisiae* strain PNY2211 (above). Forward and reverse PCR primers (N1255 and N1256, SEQ ID NOs: 251 and 252, respectively) amplified a 250 bp repeat sequence with a 5' URA3 overlap sequence tag from a genomic DNA preparation of *S. cerevisiae* strain

PNY2211. Forward and reverse PCR primers (N1257 and N1258, SEQ ID NOs: 253 and 254, respectively) amplified a 250 bp upstream homology sequence with a 5' repeat overlap sequence tag from a genomic DNA preparation of *S. cerevisiae* strain PNY2211.

Approximately 1.5 μg of the PCR amplified cassette was transformed into strain PNY1528 (above) made competent using the ZYMO Research Frozen Yeast Transformation Kit and the transformation mix plated on SE 1.0%-uracil and incubated at 30° C. for selection of cells with an integrated ymr226CΔ::URA3 cassette. Transformants appearing after 72 to 96 hours are subsequently short-streaked on the same medium and incubated at 30° C. for 24 to 48 hours. The short-streaks are screened for ymr226CΔ::URA3 by PCR, with a 5' outward facing URA3 deletion cassette-specific internal primer (N1249, SEQ ID NO: 245) paired with a flanking inward facing chromosome-specific primer (N1239, SEQ ID NO: 243) and a 3' outward-facing URA3 deletion cassette-specific primer (N1250, SEQ ID NO: 246) paired with a flanking inward-facing chromosome-specific primer (N1242, SEQ ID NO: 244). A positive PNY1528 ymr226CΔ::URA3 PCR screen resulted in 5' and 3' PCR products of 598 and 726 bp, respectively.

Three positive PNY1528 ymr226CΔ::URA3 clones were picked and cultured overnight in a YPE 1% medium of which 100 μL was plated on YPE 1%+5-FOA for marker removal. Colonies appearing after 24 to 48 hours were PCR screened for marker loss with 5' and 3' chromosome-specific primers (N1239 and N1242). A positive PNY1528 ymr226CA markerless PCR screen resulted in a PCR product of 801 bp. Multiple clones were obtained and one was designated PNY2237.

PNY2238 and PNY2243 (ALD6 Deletion Strains)

A vector was designed to replace the ALD6 coding sequence with a Cre-lox recyclable URA3 selection marker. Sequences 5' and 3' of ALD6 were amplified by PCR (primer pairs N1179 and N1180 and N1181 and N1182, respectively; SEQ ID NOs: 237, 238, 239, and 240, respectively). After cloning these fragments into TOPO vectors (Invitrogen Cat. No. K2875-J10) and sequencing (M13 forward (SEQ ID NO:269) and reverse (SEQ ID NO:270) primers), the 5' and 3' flanks were cloned into pLA33 (pUC19::loxP::URA3::loxP) (SEQ ID NO:268) at the EcoRI and SphI sites, respectively. Each ligation reaction was transformed into *E. coli* Stb13 cells, which were incubated on LB Amp plates to select for transformants. Proper insertion of sequences was confirmed by PCR (primers M13 forward (SEQ ID NO: 269) and N1180 (SEQ ID NO:238) and M13 reverse (SEQ ID NO:270) and N1181 (SEQ ID NO:239), respectively).

The vector described above (pUC19::ald6Δ::loxP-URA3-loxP) was linearized with AhdI and transformed into PNY1528 and PNY2237 using the standard lithium acetate method (except that incubation of cells with DNA was extended to 2.5 h). Transformants were obtained by plating on synthetic complete medium minus uracil that provided 1% ethanol as the carbon source. Patched transformants were screened by PCR to confirm the deletion/integration, using primers N1212 (SEQ ID NO: 241) and N1180 (5' end) (SEQ ID NO: 238) and N1181 (SEQ ID NO: 239) and N1213 (SEQ ID NO: 242) (3' end). A plasmid carrying Cre recombinase (pRS423::GAL1p-Cre=SEQ ID No. 271) was transformed into the strain using histidine marker selection, Transformants were passaged on YPE supplemented with 0.5% galactose. Colonies were screened for resistance to 5-FOA (loss of URA3 marker) and for histidine auxotrophy (loss of the Cre plasmid). Proper removal of the URA3 gene via the flanking loxP sites was confirmed by PCR (primers N1262 and N1263, SEQ ID NOs: 255 and 256, respectively). Additionally, primers internal to the ALD6 gene (N1230 and N1231; SEQ ID NOs: 261 and 262, respectively) were used to insure that no merodiploids were present. Finally, ald6Δ::loxP clones were screened by PCR to confirm that a translocation between ura3Δ::loxP (N1228 and N1229, SEQ ID NOs: 259 and 260) and gpd2Δ::loxP (N1223 and N1225, SEQ ID NOs: 257 and 258) had not occurred. Two positive clones were identified from screening of transformants of PNY1528, Clone B has been designated PNY2243. Three positive clones were identified from screening transformants of PNY2237. Clones E and K were both assessed for isobutanol production at small scale (below). Although statistically identical in most parameters, Clone E was selected (PNY2238) for further development.

Example 10

Isobutanol Pathway Plasmids

The purpose of this example is to describe construction or modification of isobutanol pathway plasmids for production of isobutanol in host strains.

pYZ067 (SEQ ID NO:374) was constructed to contain the following chimeric genes: 1) the coding region of the ilvD gene from *S. mutans* UA159 with a C-terminal Lumio tag expressed from the yeast FBA1 promoter followed by the FBA1 terminator for expression of dihydroxy acid dehydratase, 2) the coding region for horse liver ADH expressed from the yeast GPM1 promoter followed by the ADH1 terminator for expression of alcohol dehydrogenase, and 3) the coding region of the KivD gene from *Lactococcus lactis* expressed from the yeast TDH3 promoter followed by the TDH3 terminator for expression of ketoisovalerate decarboxylase.

pYZ067ΔkivDΔhADH (SEQ ID NO: 385) was constructed from pYZ067 (SEQ ID NO: 374) by deleting the promoter-gene-terminator cassettes for both kivD and adh. pYZ067 was digested with BamHI and SacI (New England BioLabs; Ipswich, Mass.), and the 7934 bp fragment was purified on an agarose gel followed by a Gel Extraction kit (Qiagen; Valencia, Calif.). The isolated fragment of DNA was treated with DNA Polymerase I, Large (Klenow) Fragment (New England BioLabs; Ipswich, Mass.) and then self-ligated with T4 DNA ligase and used to transform competent TOP10 *Escherichia coli* (Invitrogen; Carlsbad, Calif.). Plasmids from transformants were isolated and checked for the proper deletion by sequence analysis. A correct plasmid isolate was designated pYZ067ΔkivDΔhADH.

pYZ067ΔkivDΔilvD (SEQ ID NO: 772) was constructed to contain a chimeric gene having the coding region of the adh gene from horse liver (nt position 3148-2021), codon optimized for expression in *Saccharomyces cerevisiae*, expressed from the yeast GPM promoter (nt 3916-3160) and followed by the ADH1 terminator (nt 2012-1697) for expression of ADH. pYZ067DkivDDilvD was constructed from pYZ067 by deleting the promoter-gene-terminator cassettes for both kivD and ilvD. pYZ067 was digested with AatII and SacI (New England BioLabs; Ipswich, Mass.) and the 10196 bp fragment was purified on an agarose gel followed by a Gel Extraction kit (Qiagen; Valencia, Calif.). The isolated fragment of DNA was treated with DNA Polymerase I, Large (Klenow) Fragment (New England BioLabs; Ipswich, Mass.) and then self-ligated with T4 DNA ligase. The resulting plasmid was then digested with NgoMIV and BamHI (New England BioLabs; Ipswich, Mass.) and the 7533 bp fragment was purified on an agarose gel followed by a Gel Extraction

kit (Qiagen; Valencia, Calif.). The isolated fragment of DNA was treated with DNA Polymerase I, Large (Klenow) Fragment (New England BioLabs; Ipswich, Mass.) and then self-ligated with T4 DNA ligase. Plasmids were isolated and checked for the proper deletions by sequence analysis. A correct plasmid isolate was designated pYZ067DkivDDilvD.

pK9G9.OLE1p.ilvD (SEQ ID NO: 773), derived from pYZ090 (SEQ ID NO: 195), was constructed to contain a chimeric gene having the coding region of the ilvD gene from *Streptococcus mutans* (nt position 5377-3641) expressed from the yeast OLE1 promoter (nt 5986-5387) and followed by the FBA1 terminator (nt 3632-3320) for expression of DHAD, and a chimeric gene having the coding region of the variant K9G9 of the ilvC gene from *Anaerostipes caccae* (nucleic acid and amino acid SEQ ID NOs: 774 and 647) (nt 1628-2659) expressed from the yeast ILV5 promoter (nt 427-1620) and followed by the ILV5 terminator (nt 2685-3307) for expression of KARI. Construction of the plasmid was as follows. The chimeric gene from plasmid pYZ067 having the coding region of the ilvD gene from *Streptococcus mutans* expressed from the yeast FBA1 promoter and followed by the FBA1 terminator was ligated into pYZ090 after digestion with restriction enzymes NgoMIV and BamHI. The alsS coding region and 280 bp from the 3' end of the CUP1 promoter was deleted from the resulting plasmid by digesting with the restriction enzymes SpeI and Pad and self-ligating the resulting large DNA fragment. The yeast FBA1 promoter upstream of ilvD was removed from the resulting plasmid by digesting with the restriction enzymes NgoMIV and PmlI and was replaced with the yeast OLE1 promoter amplified with primers pOLE1-NgoMI (SEQ ID NO: 775) and pOLE1-PmlI (SEQ ID NO: 776). The coding region of the ilvC gene from *Lactococcus lactis* was deleted from the resulting plasmid by digestion with restriction enzymes PmeI and SfiI followed by gel purification of the large DNA fragment. The coding region of the variant K9G9 ilvC gene (SEQ ID NO: 777) from *Anaerostipes caccae* was digested out of pLH701 (SEQ ID NO: 778) with PmeI and SfiI and gel purified. The two DNA fragments were ligated to generate pK9G9.OE1p.ilvD.

Example 11

Construction of PNY2240 and PNY2242

Strain PNY2240 was derived from PNY2211 after transformation with plasmids pLH702 (SEQ ID NO: 181) and pBP915 (SEQ ID NO: 182). Transformants were plated on synthetic complete medium without histidine or uracil (1% ethanol as carbon source). Transformants were patched to the same medium containing, instead, 2% glucose and 0.05% ethanol as carbon sources. Three patches were used to inoculate liquid medium (synthetic complete minus uracil with 0.3% glucose and 0.3% ethanol as carbon sources). To test isobutanol production, liquid cultures were sub-cultured into synthetic complete medium minus uracil containing 2% glucose and 0.05% ethanol as carbon sources that also contained BME vitamin mix (Sigma Cat. No. B6891). Cultures were incubated in sealed serum vials (10 ml medium in 15 ml vials) at 30° C. with shaking (250 rpm in an Infors Multitron shaker). After 48 hours, culture medium was filtered (Spin-X column) and analyzed by HPLC (as described in US App. Pub. No. 2007/0092957, which is incorporated herein by reference in its entirety). One clone was designated PNY2240.

Strain PNY2242 was derived from PNY2238 after transformation with plasmids pLH702 (SEQ ID NO: 181) and pYZ067ΔkivDΔhADH (described herein above). Transformants were plated on synthetic complete medium without histidine or uracil (1% ethanol as carbon source). Transformants were patched to the same medium containing, instead, 2% glucose and 0.05% ethanol as carbon sources. Three patches were tested for isobutanol production, as described above. All three performed similarly in terms of glucose consumption and isobutanol production. One clone was designated PNY2242 and was further characterized under fermentation conditions, as described herein below.

Example 12

Construction of *Saccharomyces cerevisiae* Strain BP1064 (PNY1503)

The strain BP1064 was derived from CEN.PK 113-7D (CBS 8340; Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre, Netherlands) and contains deletions of the following genes: URA3, HIS3, PDC1, PDC5, PDC6, and GPD2. BP1064 was transformed with plasmids pYZ090 (SEQ ID NO:195) and pLH468 (SEQ ID NO:139) to create strain NGCI-070 (BP1083; PNY1504).

Deletions, which completely removed the entire coding sequence, were created by homologous recombination with PCR fragments containing regions of homology upstream and downstream of the target gene and either a G418 resistance marker or URA3 gene for selection of transformants. The G418 resistance marker, flanked by loxP sites, was removed using Cre recombinase (pRS423::PGAL1-cre; SEQ ID NO: 271). The URA3 gene was removed by homologous recombination to create a scarless deletion, or if flanked by loxP sites was removed using Cre recombinase.

URA3 Deletion

To delete the endogenous URA3 coding region, a ura3::loxP-kanMX-loxP cassette was PCR-amplified from pLA54 template DNA (SEQ ID NO:386). pLA54 contains the *K. lactis* TEF1 promoter and kanMX marker, and is flanked by loxP sites to allow recombination with Cre recombinase and removal of the marker. PCR was done using Phusion DNA polymerase and primers BK505 and BK506 (SEQ ID NOs: 294 and 295). The URA3 portion of each primer was derived from the 5° region upstream of the URA3 promoter and 3° region downstream of the coding region such that integration of the loxP-kanMX-loxP marker resulted in replacement of the URA3 coding region. The PCR product was transformed into CEN.PK 113-7D using standard genetic techniques (Methods in Yeast Genetics, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp. 201-202) and transformants were selected on YPD containing G418 (100 μg/ml) at 30° C. Transformants were screened to verify correct integration by PCR using primers LA468 and LA492 (SEQ ID NOs:296 and 297) and designated CEN.PK 113-7D Δura3::kanMX.

HIS3 Deletion

The four fragments for the PCR cassette for the scarless HIS3 deletion were amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs; Ipswich, Mass.) and CEN.PK 113-7D genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen; Valencia, Calif.). HIS3 Fragment A was amplified with primer oBP452 (SEQ ID NO:298) and primer oBP453 (SEQ ID NO:299), containing a 5' tail with homology to the 5' end of HIS3 Fragment B. HIS3 Fragment B was amplified with primer oBP454 (SEQ ID NO:300), containing a 5' tail with homology to the 3' end of HIS3 Fragment A, and primer oBP455 (SEQ ID NO:301), containing a 5' tail with homology to the 5' end of HIS3 Fragment U. HIS3 Fragment U was amplified

with primer oBP456 (SEQ ID NO:302), containing a 5" tail with homology to the 3' end of HIS3 Fragment B, and primer oBP457 (SEQ ID NO:303), containing a 5' tail with homology to the 5' end of HIS3 Fragment C. HIS3 Fragment C was amplified with primer oBP458 (SEQ ID NO:304), containing a 5' tail with homology to the 3' end of HIS3 Fragment U, and primer oBP459 (SEQ ID NO:305). PCR products were purified with a PCR Purification kit (Qiagen). HIS3 Fragment AB was created by overlapping PCR by mixing HIS3 Fragment A and HIS3 Fragment B and amplifying with primers oBP452 (SEQ ID NO:298) and oBP455 (SEQ ID NO:301). HIS3 Fragment UC was created by overlapping PCR by mixing HIS3 Fragment U and HIS3 Fragment C and amplifying with primers oBP456 (SEQ ID NO:302) and oBP459 (SEQ ID NO:305). The resulting PCR products were purified on an agarose gel followed by a Gel Extraction kit (Qiagen). The HIS3 ABUC cassette was created by overlapping PCR by mixing HIS3 Fragment AB and HIS3 Fragment UC and amplifying with primers oBP452 (SEQ ID NO:298) and oBP459 (SEQ ID NO:305). The PCR product was purified with a PCR Purification kit (Qiagen).

Competent cells of CEN.PK 113-7D Δura3::kanMX were made and transformed with the HISS ABUC PCR cassette using a Frozen-EZ Yeast Transformation H kit (Zymo Research; Orange, Calif.). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 2% glucose at 30° C. Transformants with a his3 knockout were screened for by PCR with primers oBP460 (SEQ ID NO:306) and oBP461 (SEQ ID NO:307) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). A correct transformant was selected as strain CEN.PK 113-7D Δura3::kanMX Δhis3::URA3.

KanMX Marker Removal from the Δura3 Site and URA3 Marker Removal from the Δhis3 Site

The KanMX marker was removed by transforming CEN.PK 113-7D Δura3::kanMX Δhis3::URA3 with pRS423::PGAL1-cre (SEQ ID NO: 271,) using a Frozen-EZ Yeast Transformation H kit (Zymo Research) and plating on synthetic complete medium lacking histidine and uracil supplemented with 2% glucose at 30° C. Transformants were grown in YP supplemented with 1% galactose at 30° C. for ~6 hours to induce the Cre recombinase and KanMX marker excision and plated onto YPD (2% glucose) plates at 30° C. for recovery. An isolate was grown overnight in YPD and plated on synthetic complete medium containing 5-fluoro-orotic acid (0.1%) at 30° C. to select for isolates that lost the URA3 marker, 5-FOA resistant isolates were grown in and plated on YPD for removal of the pRS423::PGAL1-cre plasmid, Isolates were checked for loss of the KanMX marker, URA3 marker, and pRS423::PGAL1-cre plasmid by assaying growth on YPD+G418 plates, synthetic complete medium lacking uracil plates, and synthetic complete medium lacking histidine plates. A correct isolate that was sensitive to G418 and auxotrophic for uracil and histidine was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 and designated as BP857. The deletions and marker removal were confirmed by PCR and sequencing with primers oBP450 (SEQ ID NO:308) and oBP451 (SEQ ID NO:309) for Δura3 and primers oBP460 (SEQ ID NO:306) and oBP461 (SEQ ID NO:307) for Δhis3 using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen).

PDC6 Deletion

The four fragments for the PCR cassette for the scarless PDC6 deletion were amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs) and CEN.PK 113-7D genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). PDC6 Fragment A was amplified with primer oBP440 (SEQ ID NO:310) and primer oBP441 (SEQ ID NO:311), containing a 5' tail with homology to the 5' end of PDC6 Fragment B. PDC6 Fragment B was amplified with primer oBP442 (SEQ ID NO:312), containing a 5' tail with homology to the 3" end of PDC6 Fragment A, and primer oBP443 (SEQ ID NO:313), containing a 5' tail with homology to the 5' end of PDC6 Fragment U. PDC6 Fragment U was amplified with primer oBP444 (SEQ ID NO:314), containing a 5' tail with homology to the 3' end of PDC6 Fragment B, and primer oBP445 (SEQ ID NO:315), containing a 5' tail with homology to the 5' end of PDC6 Fragment C. PDC6 Fragment C was amplified with primer oBP446 (SEQ ID NO:316), containing a 5' tail with homology to the 3' end of PDC6 Fragment U, and primer oBP447 (SEQ ID NO:317). PCR products were purified with a PCR Purification kit (Qiagen), PDC6 Fragment AB was created by overlapping PCR by mixing PDC6 Fragment A and PDC6 Fragment B and amplifying with primers oBP440 (SEQ ID NO:310) and oBP443 (SEQ ID NO:313), PDC6 Fragment UC was created by overlapping PCR by mixing PDC6 Fragment U and PDC6 Fragment C and amplifying with primers oBP444 (SEQ ID NO:314) and oBP447 (SEQ ID NO:317). The resulting PCR products were purified on an agarose gel followed by a Gel Extraction kit (Qiagen). The PDC6 ABUC cassette was created by overlapping PCR by mixing PDC6 Fragment AB and PDC6 Fragment UC and amplifying with primers oBP440 (SEQ ID NO:310) and oBP447 (SEQ ID NO:317). The PCR product was purified with a PCR Purification kit (Qiagen).

Competent cells of CEN.PK 113-7D Δura3::loxP Δhis3 were made and transformed with the PDC6 ABUC PCR cassette using a Frozen-EZ Yeast Transformation II kit (Zymo Research). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 2% glucose at 30° C. Transformants with a pdc6 knockout were screened for by PCR with primers oBP448 (SEQ ID NO:318) and oBP449 (SEQ ID NO:319) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). A correct transformant was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6::URA3.

CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6::URA3 was grown overnight in YPD and plated on synthetic complete medium containing 5-fluoro-orotic acid (0.1%) at 30° C. to select for isolates that lost the URA3 marker. The deletion and marker removal were confirmed by PCR and sequencing with primers oBP448 (SEQ ID NO:318) and oBP449 (SEQ ID NO:319) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The absence of the PDC6 gene from the isolate was demonstrated by a negative PCR result using primers specific for the coding sequence of PDC6, oBP554 (SEQ ID NO:320) and oBP555 (SEQ ID NO:321). The correct isolate was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 and designated as BP891.

PDC1 Deletion ilvDSm Integration

The PDC1 gene was deleted and replaced with the ilvD coding region from *Streptococcus mutans* ATCC #700610. The A fragment followed by the ilvD coding region from *Streptococcus mutans* for the PCR cassette for the PDC1 deletion-ilvDSm integration was amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs) and NYLA83 genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). NYLA83 is a strain which carries the PDC1 deletion-ilvDSm integration described in U.S. Patent Application Publication No. 200910305363, which is herein incorporated by reference in its entirety. PDC1 Fragment A-ilvDSm (SEQ ID NO:322) was amplified with primer oBP513 (SEQ ID NO:326) and primer oBP515

(SEQ ID NO:327), containing a 5' tail with homology to the 5' end of PDC1 Fragment B. The B, U, and C fragments for the PCR cassette for the PDC1 deletion-ilvDSm integration were amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs) and CEN.PK 113-7D genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). PDC1 Fragment B was amplified with primer oBP516 (SEQ ID NO:328) containing a 5' tail with homology to the 3' end of PDC1 Fragment A-ilvDSm, and primer oBP517 (SEQ ID NO:329), containing a 5' tail with homology to the 5' end of PDC1 Fragment U. PDC1 Fragment U was amplified with primer oBP518 (SEQ ID NO:330), containing a 5° tail with homology to the 3' end of PDC1 Fragment B, and primer oBP519 (SEQ ID NO:331), containing a 5' tail with homology to the 5' end of PDC1 Fragment C. PDC1 Fragment C was amplified with primer oBP520 (SEQ ID NO:332), containing a 5° tail with homology to the 3' end of PDC1 Fragment U, and primer oBP521 (SEQ ID NO:333). PCR products were purified with a PCR Purification kit (Qiagen). PDC1 Fragment A-ilvDSm-B was created by overlapping PCR by mixing PDC1 Fragment A-ilvDSm and PDC1 Fragment B and amplifying with primers oBP513 (SEQ ID NO:326) and oBP517 (SEC) ID NO:329). PDC1 Fragment UC was created by overlapping PCR by mixing PDC1 Fragment U and PDC1 Fragment C and amplifying with primers oBP518 (SEQ ID NO:330) and oBP521 (SEQ ID NO:333). The resulting PCR products were purified on an agarose gel followed by a Gel Extraction kit (Qiagen). The PDC1 A-ilvDSm-BUC cassette (SEQ ID NO:323) was created by overlapping PCR by mixing PDC1 Fragment A-ilvDSm-B and PDC1 Fragment UC and amplifying with primers oBP513 (SEQ ID NO:326) and oBP521 (SEQ ID NO:333). The PCR product was purified with a PCR Purification kit (Qiagen).

Competent cells of CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 were made and transformed with the PDC1 A-ilvDSm-BUC PCR cassette using a Frozen-EZ Yeast Transformation H kit (Zymo Research). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 2% glucose at 30° C. Transformants with a pdc1 knockout ilvDSm integration were screened for by PCR with primers oBP511 (SEQ ID NO:336) and oBP512 (SEQ ID NO:337) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The absence of the PDC1 gene from the isolate was demonstrated by a negative PCR result using primers specific for the coding sequence of PDC1, oBP550 (SEQ ID NO:338) and oBP551 (SEQ ID NO:339). A correct transformant was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm-URA3.

CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm-URA3 was grown overnight in YPD and plated on synthetic complete medium containing 5-fluoro-orotic acid (0.1%) at 30° C. to select for isolates that lost the URA3 marker. The deletion of PDC1, integration of ilvDSm, and marker removal were confirmed by PCR and sequencing with primers oBP511 (SEQ ID NO:336) and oBP512 (SEQ ID NO:337) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The correct isolate was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm and designated as BP907.

PDC5 Deletion sadB Integration

The PDC5 gene was deleted and replaced with the sadB coding region from *Achromobacter xylosoxidans* (the sadB gene is described in U.S. Patent Appl. No. 2009/0269823, which is herein incorporated by reference in its entirety). A segment of the PCR cassette for the PDC5 deletion-sadB integration was first cloned into plasmid pUC19-URA3MCS.

pUC19-URA3MCS is pUC19 based and contains the sequence of the URA3 gene from *Saccharomyces cerevisiae* situated within a multiple cloning site (MCS). pUC19 contains the pMB1 replicon and a gene coding for beta-lactamase for replication and selection in *Escherichia coli*. In addition to the coding sequence for URA3, the sequences from upstream and downstream of this gene were included for expression of the URA3 gene in yeast. The vector can be used for cloning purposes and can be used as a yeast integration vector.

The DNA encompassing the URA3 coding region along with 250 bp upstream and 150 bp downstream of the URA3 coding region from *Saccharomyces cerevisiae* CEN.PK 113-7D genomic DNA was amplified with primers oBP438 (SEQ ID NO:334), containing BamHI, AscI, PmeI, and FseI restriction sites, and oBP439 (SEQ ID NO:335), containing XbaI, PacI, and NotI restriction sites, using Phusion High-Fidelity PCR Master Mix (New England BioLabs). Genomic DNA was prepared using a Gentra Puregene Yeast/Bact kit (Qiagen). The PCR product and pUC19 (SEQ ID NO:325) were ligated with T4 DNA ligase after digestion with BamHI and XbaI to create vector pUC19-URA3MCS. The vector was confirmed by PCR and sequencing with primers oBP264 (SEQ ID NO:342) and oBP265 (SEQ ID NO:343).

The coding sequence of sadB and P005 Fragment B were cloned into pUC19-URA3MCS to create the sadB-BU portion of the PDC5 A-sadB-BUC PCR cassette. The coding sequence of sadB was amplified using pLH468-sadB (SEQ ID NO:359) as template with primer oBP530 (SEQ ID NO:344), containing an AscI restriction site, and primer oBP531 (SEQ ID NO:345), containing a 5' tail with homology to the 5' end of PDC5 Fragment B. PDC5 Fragment B was amplified with primer oBP532 (SEQ ID NO:346), containing a 5" tail with homology to the 3' end of sadB, and primer oBP533 (SEQ ID NO:347), containing a PmeI restriction site. PCR products were purified with a PCR Purification kit (Qiagen), sadB-PDC5 Fragment B was created by overlapping PCR by mixing the sadB and PDC5 Fragment B PCR products and amplifying with primers oBP536 (SEQ ID NO:344) and oBP533 (SEQ ID NO:347). The resulting PCR product was digested with AscI and PmeI and ligated with T4 DNA ligase into the corresponding sites of pUC19-URA3MCS after digestion with the appropriate enzymes. The resulting plasmid was used as a template for amplification of sadB-Fragment B-Fragment U using primers oBP536 (SEQ ID NO:348) and oBP546 (SEQ ID NO:349), containing a 5' tail with homology to the 5' end of PDC5 Fragment C. PDC5 Fragment C was amplified with primer oBP547 (SEQ ID NO:350) containing a 5' tail with homology to the 3' end of PDC5 sadB-Fragment B-Fragment U, and primer oBP539 (SEQ ID NO:351). PCR products were purified with a PCR Purification kit (Qiagen). PDC5 sadB-Fragment B-Fragment U-Fragment C was created by overlapping PCR by mixing PDC5 sadB-Fragment B-Fragment U and PDC5 Fragment C and amplifying with primers oBP536 (SEQ ID NO:348) and oBP539 (SEQ ID NO:351). The resulting PCR product was purified on an agarose gel followed by a Gel Extraction kit (Qiagen). The PDC5 A-sadB-BUC cassette (SEQ ID NO:324) was created by amplifying PDC5 sadB-Fragment B-Fragment U-Fragment C with primers oBP542 (SEQ ID NO:352), containing a 5' tail with homology to the 50 nucleotides immediately upstream of the native PDC5 coding sequence, and oBP539 (SEQ ID NO:351). The PCR product was purified with a PCR Purification kit (Qiagen).

Competent cells of CEN.PK 113-70 Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm were made and transformed with the

PDC5 A-sadB-BUC PCR cassette using a Frozen-EZ Yeast Transformation H kit (Zymo Research). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 1% ethanol (no glucose) at 30 C. Transformants with a pdc5 knockout sadB integration were screened for by PCR with primers oBP540 (SEQ ID NO:353) and oBP541 (SEQ ID NO:354) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The absence of the PDC5 gene from the isolate was demonstrated by a negative PCR result using primers specific for the coding sequence of PDC5, oBP552 (SEQ ID NO:355) and oBP553 (SEQ ID NO:356). A correct transformant was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm Δpdc5::sadB-URA3.

CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm Δpdc5::sadB-URA3 was grown overnight in YPE (1% ethanol) and plated on synthetic complete medium supplemented with ethanol (no glucose) and containing 5-fluoro-orotic acid (0.1%) at 30 C to select for isolates that lost the URA3 marker. The deletion of PDC5, integration of sadB, and marker removal were confirmed by PCR with primers oBP540 (SEQ ID NO:353) and oBP541 (SEQ ID NO:354) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The correct isolate was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm Δpdc5::sadB and designated as BP913.

GPD2 Deletion

To delete the endogenous GPD2 coding region, a gpd2::loxP-URA3-loxP cassette (SEQ ID NO:361) was PCR-amplified using loxP-URA3-loxP PCR (SEQ ID NO:360) as template DNA. loxP-URA3-loxP contains the URA3 marker from (ATCC #77107) flanked by loxP recombinase sites. PCR was done using Phusion DNA polymerase and primers LA512 and LA513 (SEQ ID NOs:340 and 341). The GPD2 portion of each primer was derived from the 5' region upstream of the GPD2 coding region and 3° region downstream of the coding region such that integration of the loxP-URA3-loxP marker resulted in replacement of the GPD2 coding region. The PCR product was transformed into BP913 and transformants were selected on synthetic complete media lacking uracil supplemented with 1% ethanol (no glucose). Transformants were screened to verify correct integration by PCR using primers oBP582 and AA270 (SEQ ID NOs:357 and 358).

The URA3 marker was recycled by transformation with pRS423::PGAL1-cre (SEQ ID NO:271) and plating on synthetic complete media lacking histidine supplemented with 1% ethanol at 30 C. Transformants were streaked on synthetic complete medium supplemented with 1% ethanol and containing 5-fluoro-orotic acid (0.1%) and incubated at 300 to select for isolates that lost the URA3 marker. 5-FOA resistant isolates were grown in YPE (1% ethanol) for removal of the pRS423::PGAL1-cre plasmid. The deletion and marker removal were confirmed by PCR with primers oBP582 (SEQ ID NO:357) and oBP591 (SEQ ID NO:362). The correct isolate was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm Δpdc5::sadB Δgpd2::loxP and designated as BP1064 (PNY1503).

Example 13

Construction of PNY2204 and Isobutanol Pathway Plasmids

The purpose of this example is to describe construction of a vector to enable integration of a gene encoding acetolactate synthase into the naturally occurring intergenic region between the PDC1 and TRX1 coding sequences in Chromosome XII. Strains resulting from the use of this vector are also described.

Construction Of Integration Vector pUC19-kan::pdc1::FBA-alsS::TRX1

The FBA-alsS-CYCt cassette was constructed by moving the 1.7 kb BbvCI/PacI fragment from pRS426::GPD::alsS::CYC (described in U.S. Pat. No. 7,851,188, which is herein incorporated by reference in its entirety) to pRS426::FBA::ILV5::CYC (described in U.S. Pat. No. 7,851,188, which is herein incorporated by reference in its entirety), which had been previously digested with BbyCI/PacI to release the ILV5 gene. Ligation reactions were transformed into *E. coli* TOP10 cells and transformants were screened by PCR using primers N98SeqF1 (SEQ ID NO:363) and N99SeqR2 (SEC) ID NO:365). The FBA-alsS-CYCt cassette was isolated from the vector using BglII and NotI for cloning into pUC19-URA3::ilvD-TRX1 (done "B") at the AflII site (Klenow fragment was used to make ends compatible for ligation). Transformants containing the alsS cassette in both orientations in the vector were obtained and confirmed by PCR using primers N98SeqF4 (SEQ ID NO:364) and N1111 (SEQ ID NO:366) for configuration "A" and N98SeqF4 (SEQ ID NO:364) and N1110 (SEQ ID NO:367) for configuration "B". A geneticin selectable version of the "A" configuration vector was then made by removing the URA3 gene (1.2 kb NotI/NaeI fragment) and adding a geneticin cassette. Klenow fragment was used to make all ends compatible for ligation, and transformants were screened by PCR to select a clone with the geneticin resistance gene in the same orientation as the previous URA3 marker using primers BK468 (SEQ ID NO:368) and N160SeqF5 (SEQ ID NO:210). The resulting clone was called pUC19-kan::pdc1::FBA-alsS::TRX1 (clone A) (SEQ ID NO:387).

Construction of alsS Integrant Strains and Isobutanol-Producing Derivatives

The pUC19-kan::pdc1::FBA-alsS integration vector described above was linearized with PmeI and transformed into PNY1507 (Example 8). PmeI cuts the vector within the cloned pdc1-TRX1 intergenic region and thus leads to targeted integration at that location (Rodney Rothstein, Methods in Enzymology, 1991, volume 194, pp. 281-301). Transformants were selected on YPE plus 50 μg/ml G418. Patched transformants were screened by PCR for the integration event using primers N160SeqF5 (SEQ ID NO:210) and oBP512 (SEQ ID NO:337). Two transformants were tested indirectly for acetolactate synthase function by evaluating the strains ability to make isobutanol. To do this, additional isobutanol pathway genes were supplied on *E. coli-yeast* shuttle vectors (pYZ090ΔalsS and pBP915, described below). One done was designated as PNY2205. The plasmid-free parent strain was designated PNY2204 (MATa ura3Δ::loxP his3Δ pdc6Δ pdc1Δ::P[PDC1]-DHAD|ilvD_Sm-PDC1t-pUC19-loxP-kanMX-loxP-P[FBA1]-ALS|alsS_.Bs-CYC1t pdc5Δ::P[PDC5]-ADH|sadB_Ax-PDC5t gpd2Δ::loxP fra2Δ adh1Δ::UAS(PGK1)P[FBA1]-kivD_Ll(y)-ADH1t).

Isobutanol Pathway Plasmids (pYZ090ΔalsS and pBP915)

pYZ090 (SEQ ID NO:195) was digested with SpeI and NotI to remove most of the CUP1 promoter and all of the alsS coding sequence and CYC terminator. The vector was then self-ligated after treatment with Klenow fragment and transformed into *E. coli* Stbl3 cells, selecting for ampicillin resistance. Removal of the DNA region was confirmed for two independent clones by DNA sequencing across the ligation junction by PCR using primer N191 (SEQ ID NO:370). The resulting plasmid was named pYZ090ΔalsS (SEQ ID NO:371). The pLH468 plasmid was constructed for expres-

sion of DHAD, KivD and HADH in yeast. pBP915 (SEQ ID NO: 182) was constructed from pLH468 (SEQ ID NO:139) by deleting the kivD gene and 957 base pairs of the TDH3 promoter upstream of kivD. pLH468 was digested with SwaI and the large fragment (12896 bp) was purified on an agarose gel followed by a Gel Extraction kit (Qiagen; Valencia, Calif.). The isolated fragment of DNA was self-ligated with T4 DNA ligase and used to transform electrocompetent TOP10 *Escherichia coli* (Invitrogen; Carlsbad, Calif.). Plasmids from transformants were isolated and checked for the proper deletion by restriction analysis with the SwaI restriction enzyme. Isolates were also sequenced across the deletion site with primers oBP556 (SEQ ID NO:372) and oBP561 (SEQ ID NO:373). A clone with the proper deletion was designated pBP915 (pLH468ΔkivD) (SEQ ID NO:182).

pYZ090 is based on the pHR81 (ATCC #87541. Manassas, Va.) backbone. pYZ090 was constructed to contain a chimeric gene having the coding region of the alsS gene from *Bacillus subtilis* (nt position 457-2172) expressed from the yeast CUP1 promoter (nt 2-449) and followed by the CYC1 terminator (nt 2181-2430) for expression of ALS, and a chimeric gene having the coding region of the ilvC gene from *Lactococcus lactis* (nt 3634-4656) expressed from the yeast ILV5 promoter (2433-3626) and followed by the ILV5 terminator (nt 4682-5304) for expression of KARL

Example 14

Isobutanol Production-PNY1910 and PNY2242

Methods:

Preparation of Inoculum Medium

1 L of inoculum medium contained: 67 g, Yeast Nitrogen Base w/o amino acids (Difco 0919-15-3); 2.8 g, Yeast Synthetic Drop-out Medium Supplement Without Histidine, Leucine, Tryptophan and Uracil (Sigma Y2001); 20 mL of 1% (w/v) L-Leucine; 4 mL of 1% (w/v) L-Tryptophan; 3 g of ethanol; 10 g of glucose.

Preparation of Defined Fermentation Medium

The volume of broth after inoculation was 800 mL, with the following final composition, per liter: 5 g ammonium sulfate, 2.8 g potassium phosphate monobasic, 1.9 g magnesium sulfate septahydrate, 0.2 mL antifoam (Sigma DF204), Yeast Synthetic Drop-out Medium Supplement without Histidine, Leucine, Tryptophan, and Uracil (Sigma Y2001), 16 mg L-leucine, 4 mg L-tryptophan, 6 mL of a vitamin mixture (in 1 L water, 50 mg biotin, 1 g Ca-pantothenate, 1 g nicotinic acid, 25 g myoinositol, 1 g thiamine chloride hydrochloride, 1 g pyridoxol hydrochloride, 0.2 g p-aminobenzoic acid) 6 mL of a trace mineral solution (in 1 L water, 15 g EDTA, 4.5 g zinc sulfate heptahydrate, 0.8 g manganese chloride dehydrate, 0.3 g cobalt chloride hexahydrate, 0.3 g copper sulfate pentahydrate, 0.4 g disodium molybdenum dehydrate, 4.5 g calcium chloride dihydrate, 3 g iron sulfate heptahydrate, 1 g boric acid, 0.1 g potassium iodide), 30 mg thiamine HCl, 30 mg nicotinic acid. The pH was adjusted to 5.2 with 2N KOH and glucose added to 10 g/L.

Preparation of Inoculum

A 125 mL shake flask was inoculated directly from a frozen vial by pipetting the whole vial culture (approx. 1 ml) into 10 mL of the inoculum medium. The flask was incubated at 260 rpm and 30° C. The strain was grown overnight until OD about 1.0 OD at λ=600 nm was determined in a Beckman spectrophotometer (Beckman, USA).

Bioreactor Experimental Design

Fermentations were carried out in 1 L Biostat B DCU3 fermenters (Sartorius, USA) with a working volume on 0.8 L. Off-gas composition was monitored by a Prima DB mass spectrometer (Thermo Electron Corp., USA). The temperature was maintained at 30 C and pH controlled at 5.2 with 2N KOH throughout the entire fermentation. Directly after inoculation with 80 mL of the inoculum, dθ was controlled by agitation at 30%, pH was controlled at 5.25, aeration was controlled at 0.2 L/min. Once OD of approximately 3 was reached, the gas was switched to N2 for anaerobic cultivation. Throughout the fermentation, glucose was maintained in excess (5-20 g/L) by manual additions of a 50% (w/w) solution.

Methods for Analyzing Cultivation Experiments

OD at λ=600 nm was determined in a spectrophotometer by pipetting a well mixed broth sample into a cuvette (CS500 VWR International, Germany). If biomass concentration of the sample exceeded the linear absorption range of the spectrophotometer (typically OD values from 0.000 to 0.300), the sample was diluted with 0.9% NaCl solution to yield values in the linear range.

Measurements of glucose, isobutanol, and other fermentation byproducts in the culture supernatant were carried out by HPLC, using a Bio-Rad Aminex HPX-87H column (Bio-Rad, USA), with refractive index (RI) and a diode array (210 nm) detectors. Chromatographic separation was achieved using 0.01 NH_2SO_4 as the mobile phase with a flow rate of 0.6 mL/min and a column temperature of 40° C. Isobutanol retention time is 32.2 minutes under these conditions. Isobutanol concentration in off-gas samples was determined by mass-spectrometer.

Results

Maximal biomass concentration measured as optical density (OD), volumetric rate of isobutanol production, final isobutanol titer, and isobutanol yield on glucose are presented in the table below. The strain PNY2242 had higher titers and faster rates than the strain PNY1910 and produced isobutanol with higher specific rate and titer. The specific rates are shown in FIG. **5**. Accumulation of the DHIV DHMB in the culture supernatant was three times higher with PNY1910 compared to the PNY2242 strain (FIG. **6**), Yield of glycerol, pyruvic acid, BDO, DHIV+DHMB*, αKIV, and isobutyric acid on glucose is shown in FIG. **7**.

*DHIV analyzed by HPLC method includes both DHIV and DHMB.

TABLE 19

Strain	Max. OD600	Rate (g/L/h)	Titer (g/L)	Yield (g/g)
PNY1910	5.0	0.16	10.9	0.25
PNY2242	5.0	0.23	16.1	0.27

Example 15

Construction of K9G9 Error Prone PCR Library

Error prone PCR of K9G9 was performed to generate a library that can be screened for variants with increases in the Km values for NADPH relative to NADH. Mutagenic PCR of K9G9 was performed with the GeneMorph® II EZClone Domain Mutagenesis Kit (Catalog #200552; Agilent Technologies, Stratagene Products Division, La Jolla, Calif.). Primers K9G9_EZ_F1 (AAA CAT GGA AGA ATG TAA GAT GGC; SEQ ID NO: 390) and K9G9_EZ_R1 (TCA GTT GTT AAT CAA CTT GTC TTC G; SEQ ID NO: 391) were commercially synthesized by Integrated DNA Technologies,

Inc (Coralville Iowa). Other than the primers, template, and ddH_2O, reagents used here were supplied with the kit indicated above. The mutagenic PCR mixture consisted of 4 μl of pHR81-Pllv5-KARI-K9.G9 (SEQ ID NO: 392) (770 ng/μg), 1.25 μl of each primer (100 ng/μl stocks), 5 μl of 10× Mutazyme II reaction buffer, 1 μl of 40 mM dNTP mix, 1.5 μl of Mutazyme II DNA polymerase, and 36 μl of ddH_2O. The following conditions were used for the PCR reaction: The starting temperature was 95° C. for 2.0 min followed by 30 heating/cooling cycles. Each cycle consisted of 95° C. for 30 sec, 48° C. for 30 sec, and 72° C. for 2.0 min. At the completion of the temperature cycling, the sample was kept at 72° C. for 10.0 min more, and then held awaiting sample recovery at 4° C. The reaction product was separated from the template via agarose gel electrophloresis (1% agarose, 1×TBE buffer) and recovered using the StrataPrep® DNA Gel Extraction Kit (Cat#400766, Agilent Technologies, Siratagene Products Division, La Jolla, Calif.) as recommended by the manufacturer.

The isolated reaction product was employed as a megaprimer to generate gene libraries in the "EZClone reaction" of the kit indicated above. Other than the megaprimer, template, and ddH_2O, reagents used here were supplied with the kit indicated above. The reaction consisted of 25 μl of the 2× EZClone enzyme mix, 4 μl of megaprimer (125 ng/μl), 2 μl of K9G9 in a pBAD.KARI vector (25 ng/μl), 3 μl of EZClone solution, and 160 of ddH_2O. The following conditions were used for the reaction: The starting temperature was 95° C. for 1.0 min followed by 30 heating/cooling cycles. Each cycle consisted of 95° C. for 50 sec, 60° C. for 50 sec, and 68° C. for 10.0 min. At the completion of the temperature cycling, the samples were kept at 72° C. for 10.0 min more, and then held awaiting sample recovery at 4° C. 1 μl of the Dpn I (10 U/μl) was added and the mixture was incubated for 4 hours at 37° C.

4 μl of the Dpn I digested "EZClone reaction" product was then transformed into 50 μl XL10-Gold® Ultracompetent *E. coli* cells (provided in the GeneMorph® H EZClone Domain Mutagenesis Kit) as recommended by the manufacturer. The transformants were spread on agar plates containing the LB medium and 100 μg/ml ampicillin (Cat#L1004, Teknova Inc. Hollister, Calif.), incubated at 37° C. overnight, and store at 4° C. These steps were repeated with 4 μl Dpn I digested "EZClone reaction" product and 50 μl cells per transformation for a total of 10 transformations. The resultant library in XL-Gold was scraped off the agar plates with a solution containing M9 salts, combined, diluted into media containing the LB medium and 100 μg/ml ampicillin, and incubated at 37° C. overnight. The library DNA was isolated from the cells with the QIAprep Spin Miniprep Kit (Catalog #2706; Qiagen, Valencia, Calif.) according to the protocol provided by the manufacturer. The amplified library was then used to transform an electro-competent strain of *E. coli* Bw25113 (ΔilvC) using a BioRad Gene Pulser II (Bio-Rad Laboratories Inc., Hercules, Calif.). The transformed clones were spread on agar plates containing the LB medium and 100 μg/ml ampicillin (#101320-154, Teknova Inc. Hollister, Calif.) and incubated at 37° C. overnight. Clones were employed for high throughput screening as described in Example 16.

Example 16

Identification of K9G9 Variants with Increased K_M for NADPH Via Screening for Diminished NADP+ Inhibition of NADH Activity

The K9G9 library described in Example 15 was screened for variants with reduced $NADP^+$ inhibition of NADH-dependent KARI activity. A K9G9 variant with reduced $NADP^+$ inhibition of activity with NADH can potentially exhibit an increase in the ratio of the K_M for NADPH to the K_M for NADH. With a specific objective to increase K_M for NADPH relative to Km for NADH, the hits from the screen were partially purified and kinetic analyses were performed to determine V_{max} and K_M parameters with NADH and with NADPH.

High Throughput Screening Assay of K9G9 Gene Library

High throughput screening of the gene libraries of mutant KARI enzymes was performed as described herein: 10× freezing medium containing 554.4 g/L glycerol, 68 mM of $(NH_4)_2SO_4$, 4 mM $MgSO_4$, 17 mM sodium citrate, 132 mM KH_2PO_4, 36 mM K_2HPO_4 was prepared with molecular pure water and filter-sterilized. Freezing medium was prepared by diluting the 10× freezing medium with the LB medium. An aliquot (200 μL) of the 1× freezing medium was used for each well of the 96-well archive plates (cat #3370, Corning Inc. Corning, N.Y.).

Clones from the LB agar plates were selected and inoculated into the 96-wed archive plates containing the freezing medium and grown overnight at 37° C. without shaking. The archive plates were then stored at −80° C. *E. coli* strain Bw25113(ΔilvC), as described in U.S. Pat. No. 8,129,162, transformed with pBAD-HisB (Invitrogen) was always used as the negative control. The positive control for the library was K9G9-KARI in *E. coli* strain Bw25113 (ΔilvC), as described in U.S. Pat. No. 8,129,162.

Clones from archive plates were inoculated into the 96-deep well plates. Each well contained 3.0 μl of cells from thawed archive plates, 200 μl of the LB medium containing 100 μg/ml ampicillin and 0.02% (w/v) arabinose as the inducer. Cells were the grown overnight at 37° C. with 80% humidity while shaking (900 rpm), harvested by centrifugation (3750 rpm, 5 min at 25° C.). (Eppendorf centrifuge, Brinkmann Instruments, Inc. Westbury, N.Y.) and the cell pellet was stored at −20° C. for later analysis.

The assay substrate, (R,S)-acetolactate, was synthesized as described by Aulabaugh and Schloss (Aulabaugh and Schloss, Biochemistry, 29: 2824-2830, 1990). All other chemicals used in the assay were purchased from Sigma. The enzymatic conversion of acetolactate to α,β-dihydroxyisovalerate by KARI was followed by measuring the oxidation of the cofactor, NADH, from the reaction at 340 nm using a plate reader (Saphire 2, Tecan, Mannedorf, Switzerland). The activity was calculated using the molar extinction coefficient of 6220 $M^{-1}cm^{-1}$ NADH.

Frozen cell pellet in deep-well plates and BugBuster (Novagen 71456, Darmstadt, Germany) were warmed up at room temperature for 30 min at the same time. 75 μl of 50% BugBuster (v/v in water) was added to each well after 30 min warm-up and cells were suspended using plate shaker. The plates with cell pellet/50% Bug Buster suspension were incubated at room temperature for 30 min. Cell lysate diluted with 75 μL d.d water, resulting in 0.5× lysate. Assays of the diluted cell free extracts were performed at 30° C. in buffer containing 2.4 mM (R/S)-acetolactate, 100 mM HEPES pH 6.8, 100 mM KCl, 10 mM $MgCl_2$, 150 μM NADH, 12.5 μL 0.5× cell lysate with or without 2.5 mM NADP+.

Identification of K9G9 Variants with Reduced NADP+ Inhibition of NADH KARI Activity

The ratio for the measured rate of NADH oxidation in the presence of NADP+ to the measured rate of NADH oxidation in the absence of NADP+ was calculated for each variant and positive control well (2 per plate). The mean and standard deviation of ratios for all of the positive control wells (104 total) were calculated.

A variant well was considered to contain an initial hit if the rate in the absence of NADP+ was greater than 0.1 OD/hr and the rate ratio was both greater than 0.45 (three standard deviations higher than the positive control mean) and less than 1. A total of 521 hits were identified from a pool of 4607 potential variants. These initial hits were consolidated, forming a smaller library for further analysis.

Secondary Screening of Initial Library Hit

The consolidated hit library was grown in biological triplicate and cell free extracts were prepared and assayed as described above. Rate ratios were then calculated for the variants and positive controls as above. Final hits that were selected for detailed kinetic analysis met the following criteria: the rate in the absence of NADP+ was greater than 0.6 OD/hr, rate ratio was greater than 0.51 and less than 1, and at least two out of three biological replicates passed the criteria. Seventeen hits were identified for kinetic analysis and streaked out on to LB plates with 100 μg/mL ampicillin added.

Sequence Analysis of K9G9 Variants

DNA sequencing of the seventeen variants identified from the secondary HTS screening was accomplished by using TempliPhi™ (GE Healthcare) with the primers pBAD-For (ATGCCATAGCATTTTTATCC; SEQ ID NO: 393) and pBAD-Rev (CTGATTTAATCTGTATCAGGCT; SEQ ID NO: 394).

TABLE 20

Amino Acid Substitutions for K9G9 Variants

Variant	Seq	Amino Acid Substitutions
878 C1	873	None identified
879 A7	874	K90M
879 C2	875	H37Q
880 A11	876	A182T, P320Q
880 B4	877	K57E
880 D11	878	K90M, A174V
881 A2	879	K90M, I133V, K282T
881 G3	880	Y53F, E74G
881 G9	881	K90E
882 B12	882	H118R
882 C10	883	G31S, R61S, C121Y, D129N, G183D
882 C7	933	E54G
882 F9	934	K90E, Q160H
882 G6	935	G55A
882 G12	936	V142L, S285Y
883 C4	937	A170V
883 G9	938	L197M, K310M

Kinetic Analysis of Partially Purified Variant Protein

E. coli strain Bw25113 (ΔilvC), as described in U.S. Pat. No. 8,129,162, was used to express the seventeen variants and positive control K9G9. Strains were grown for 8 hours in 10 mL of LB broth (#46-060-CM, Mediatech, Manassas, Va.) containing 100 μg/mL ampicillin at 37° C. with shaking in 125 mL baffled, vented filtered lid flasks. 200 μL of this culture was used to inoculate 100 mL LB broth with 100 μg/mL ampicillin and 0.2% (w/v) arabinose added. These cultures were grown for 16 to 18 hours at 37° C. with shaking in 500 mL baffled, vented filtered lid flasks. Cells were harvested in a 20 mL and two 40 mL aliquots, supernatants were decanted and the pellets were frozen at −80° C.

To partially purify the protein, the cell pellet corresponding with the mL cell culture harvest was thawed and resuspended in 1 mL Bug Buster Master Mix (Novagen 71456, Darmstadt, Germany). The cell suspension was incubated at room temperature for 15 minutes followed by 15 minute incubation at 60° C. to denature the heat liable proteins. Cell debris and denatured proteins were pelleted by centrifugation for 30 minutes at 4° C. Supernatant containing the heat stable cytosolic protein, including K9G9 and variants, was recovered and stored at 4° C.

The total protein of the heat stable cytosolic protein fraction was measured by the Bradford Assay using Coomaisse Plus (Thermo Scientific #23238, Rockford, Ill.). BSA was employed as the standard. The concentration of protein was measured by determining the absorbance at 595 nm using a Cary 300 spectrophotometer (Agilent Technologies, Wilmington, Del.).

To determine V_{max} and K_M values for NADH and NADPH, the partially purified proteins were assayed at various concentrations of NADH (0, 16.4, 32.8, 65.7, 98.5, 164.3 and 246.5 μM) and NADPH (0, 12.8, 25.6, 51.2, 76.8 and 128 WO), Assays were conducted at 30° C. in 100 mM HEPES (pH 6.8), 10 mM $MgCl_2$, 100 mM KCl and 4.8 mM R/S-acetolactate. Between 0.1 to 0.35 mg/mL total protein was added to the assay. The rate of conversion of S-acetolactate to DHIV was measured via monitoring the oxidation of NAD(P)H at 340 nm using a Cary 300 spectrophotometer (Agilent Technologies, Wilmington, Del.). The activity was calculated using the molar extinction coefficient of 6220 K^3 cm^{-1}. V_{max} and K_m values were calculated by plotting specific activity (U/mg) vs. cofactor concentration and the data were fit to the Michaelis-Menten equation using Kaleidagraph software (Synergy, Reading, Pa.).

TABLE 21

Kinetic Values for Partially Purified K9G9 Variants as Determined via NAD(P)H Consumption Assays

Variant	V_{max} NADPH, U/mg	K_m NADPH, μM	V_{max}/K_m NADPH, L/min * mg	V_{max} NADH, U/mg	K_m NADH, μM	V_{max}/K_m NADH, L/min * mg
K9G9	1.53	45.5	0.034	1.09	67.4	0.016
878 C1	0.75	42.2	0.018	0.62	107.8	0.006
879 A7	2.51	546	0.005	1.44	263	0.006
879 C2	1.27	103	0.012	1.23	187	0.007
880 A11	0.72	86.9	0.008	0.51	117	0.004
880 B4	1.23	233	0.005	1.14	133	0.009
880 D11	1.38	130	0.011	1.50	232	0.006
881 A2	0.88	93.5	0.009	1.13	166.8	0.007
881 G3	0.69	99.2	0.007	0.69	61.8	0.011
881 G9	1.03	158	0.007	0.96	310	0.003
882 B12	0.87	30.3	0.029	0.49	78.9	0.006
882 C10	0.71	34.1	0.021	0.56	97.9	0.006
882 C7	1.62	45.3	0.036	0.96	75.6	0.013

TABLE 21-continued

Kinetic Values for Partially Purified K9G9 Variants as Determined via NAD(P)H Consumption Assays

Variant	V_{max} NADPH, U/mg	K_m NADPH, μM	V_{max}/K_m NADPH, L/min * mg	V_{max} NADH, U/mg	K_m NADH, μM	V_{max}/K_m NADH, L/min * mg
882 F9	1.39	256	0.005	1.19	335	0.004
882 G6	0.95	47.4	0.020	0.74	98.7	0.007
882 G12	1.06	63.5	0.017	0.75	81.2	0.009
883 C4	1.26	46.8	0.027	0.67	83.9	0.008
883 G9	1.26	38	0.033	1.01	71.9	0.014

Example 17

Manual Recombination of K9 KARI Variants Via Site Directed Mutagenesis

Site directed mutagenesis of the K9G9 derivatives K9JB4 and K9JG3 (identified in Example 16 as 880 B4 and 881 G3, respectively) was performed to incorporate other amino acid changes described in the examples. The initial step was to add to the N87P substitution, which is described in Example 5. Mutations were introduced into the KARI genes with primers N87PC1 (CTGACATCATTATGATCTTGATCCCA-GATGAAAAGCAGGCTACCATG TAC; SEQ ID NO: 395) and N87PC1r (GTACATGGTAGCCTGCTTTTCATCTGG-GATCAAGATCATAATGATGT CAG; SEQ ID NO: 396), employing the QuikChange® II Site-Directed Mutagenesis Kit (Catalog #200523; Agilent Technologies, Stratagene Products Division, La Jolla, Calif.). Except for the primers, templates, and ddH_2O, all reagents used here were supplied with the kit indicated above. Primers were commercially synthesized by Integrated DNA Technologies, Inc (Coralville Iowa). Templates were K9 KARI variants in *E. coli* vectors (pBAD.KARI). For mutagenesis of K9JB4, the reaction mixture contained 1 μl K9JB4 (50 ng/μl), 1 μl of each primer (150 ng/ul), 5 μl of 10× reaction buffer, 1 μl of dNTP mix, 1 μl of Pfu Ultra HF DNA polymerase, and 40 μl of ddH_2O. For, the K9JG3 reaction mixture, 1 μl K9JB4 (50 ng/ul) was substituted with 1 μl K9JG3 (50 ng/μl). The following conditions were used for both reactions: The starting temperature was 95° C. for 30 sec followed by 16 heating/cooling cycles. Each cycle consisted of 95° C. for 30 sec, 55° C. for 30 sec, and 68° C. for 5.0 min. At the completion of the temperature cycling, the samples held awaiting sample recovery at 4° C. 1 μl of the Dpn I (10 U/μl) was added to each reaction and the mixtures were incubated for 1 hour at 37° C.

2 μl of each mutagenic reaction was transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen, Catalog #C404003) according to the manufacturer's instructions. The transformants were spread on agar plates containing the LB medium and 100 μg/ml ampicillin (Cat#L1004, Teknova Inc. Hollister, Calif.) and incubated at 37° C. overnight. Multiple transformants were then selected for TempliPhi™ (GE Healthcare) based DNA sequencing employing primers pBAD-For (ATGCCATAGCATTTTTATCC; SEQ ID NO: 393) and pBAD-Rev (CTGATTTAATCTGTAT-CAGGCT; SEQ ID NO: 394). Transformants with confirmed KARI sequences were inoculated into LB medium containing 100 μg/ml ampicillin and incubated at 33° C. with shaking at 225 rpm. Plasmid DNA was isolated from the cells with the QIAprep Spin Miniprep Kit (Catalog #2706; Qiagen, Valencia, Calif.) according to the protocol provided by the manufacturer. The resultant clones K9JB4P and K9JG3P were derived from K9JB4 and K9JG3, respectively.

Additional Site Directed Mutagenesis was Performed as Described Above With Modifications.

Variant K9JA1 was derived from K9JG3P employing primers oK57E1 (GGTTTATTCGAAGGTGCGGAG-GAGTGGAAAAGAGCTG; SEQ ID NO: 397) and oK57E1r (CAGCTCTTTTCCACTCCTCCGCACCTTC-GAATAAACC; SEQ ID NO: 398). The mutagenesis reaction contained 1 μl K9JG3P (50 ng/μl), 1 μl of each primer (150 ng/ul), 5 μl of 10× reaction buffer, 1 μl of dNTP mix, 1 μl of PfuUltra HF DNA polymerase, and 40 μl of ddH_2O. Liquid cultures for *E. coli* transformants were incubated at 37° C. instead of 33° C.

Variant K9SB2 was derived from K9JB4P employing primers oY53F1 (GTAACGTTATCATTGGTTTATAC-GAAGGTGCGGAGGAG; SEQ ID NO: 399) and oY53F1r (CTCCTCCGCACCTTCGAATAAACCAAT-GATAACGTTAC; SEQ ID NO: 400). The mutagenesis reaction contained 1 μl K9JB4P (50 ng/μl), 1 μl of each primer (150 ng/ul), 5 μl of 10× reaction buffer, 1 μl of dNTP mix, 1 μl of PfuUltra HF DNA polymerase, and 40 μl of ddH2O. Liquid cultures for *E. coli* transformants were incubated at 37° C. instead of 33° C.

Variant K9SB2-K90L was derived from K9SB2 employing primers oK90L1(GATCTTGATCCCAGATGAATTG-CAGGCTACCATGTACAAAAA C; SEQ ID NO: 401) and oK90L1 r (GTT TTT GTA CAT GGT AGO CTG CAA TTC ATC TGG GAT CAA GAT C; SEQ ID NO: 402). The mutagenesis reaction contained 2.5 μl K9SB2 (50 ng/μl), 1 μl of each primer (150 ng/ul), 5 μl of 10× reaction buffer, 1 μl of dNTP mix, 1 μl of PfuUltra HF DNA polymerase, and 38.5 μl of ddH_2O. For the heating/cooling cycles, the step of 55° C. for 30 sec was increased to 1 min. Liquid cultures for *E. coli* transformants were incubated at 37° C. instead of 33° C.

Variant K9SB2-K90M was derived from K9SB2 employing primers oK90M1 (CTTGATCCCAGATGAAATGCAG-GCTACCATGTACAAAAAC; SEQ ID NO: 403) and oK90M1r (GTT TTT GTA CAT GGT AGO CTG CAT TTC ATC TGG GAT CAA G; SEQ ID NO: 404). The mutagenesis reaction contained 2.5 μl K9SB2 (50 ng/μl), 1 μl of each primer (150 ng/ul), 5 μl of 10× reaction buffer, 1 μl of dNTP mix, 1 μl of PfuUltra HF DNA polymerase, and 38.5 μl of ddH_2O. For the heating/cooling cycles, the step of 55° C. for 30 sec was increased to 1 min. Liquid cultures for *E. coli* transformants were incubated at 37° C. instead of 33° C.,

TABLE 22

Amino Acid Substitutions of K9G9 Variants and Combinations

Variant	Amino Acid Seq ID No:	Nucleic Acid SEQ ID NO:	Amino Acid Substitutions
K9JB4	417	418	S56A, K57E, S58E
K9JB4P	419	420	S56A, K57E, S58E, N87P
K9JG3	421	422	Y53F, S56A, S58E, E74G
K9JG3P	423	424	Y53F, S56A, S58E, E74G, N87P
K9JA1	425	426	Y53F, S56A, K57E, S58E, E74G, N87P
K9SB2	427	428	Y53F, S56A, K57E, S58E, N87P
K9SB2-K90L	429	430	Y53F, S56A, K57E, S58E, N87P, K90L
K9SB2-K90M	431	432	Y53F, S56A, K57E, S58E, N87P, K90M

Example 18

Kinetic Characterization of Purified K9G9 Derivatives with Increased Ratios of K_M NADPH to K_M NADH

K9G9 and variants were overexpressed in *E. coli* strain Bw25113 (ΔilvC), as described in U.S. Pat. No. 8,129,162, and purified in order to obtain a more accurate determination of cofactor affinity and maximum velocity.

For expression and characterization, *E. coli* plasmids (pBAD.KARI) were used to transform an electro-competent strain of *E. coli* Bw25113 (ΔivC) as described in U.S. Pat. No. 8,129,162, using a BioRad Gene Pulser II (Bio-Rad Laboratories Inc., Hercules, Calif.). The transformed clones were spread on agar plates containing the LB medium and 100 μg/ml ampicillin (#101320-154, Teknova Inc. Hollister, Calif.) and incubated at 37° C. overnight. A single transformant for each strain was streaked out onto LB plates with 100 μg/mL ampicillin. A single colony from each of these plates was used to inoculate 10 mL LB broth with 100 μg/mL ampicillin. These cultures were grown for 8 hours at 37° C. with shaking in 125 mL baffled flasks with vented, filtered lids. 200 μL of this culture was used to inoculate two 500 mL baffled flasks with filtered vented lids containing LB broth with 100 μg/mL ampicillin and 0.2% (w/v) arabinose. The expression cultures were grown for 16-18 hours at 37° C. with shaking. Cells were harvested in 40 mL aliquots via centrifugation; the supernatant was discarded and cell pellets were frozen at −80° C. until purification.

K9G9 and all variants were purified using the same process. Two cell pellets, representing 40 mL cell culture aliquots each, were resuspended in 4 mL Bug Buster Master Mix (Novagen 71456, Darmstadt, Germany) and incubated for 15 minutes at room temperature followed by 15 minutes at 60° C. Denatured proteins and cell debris was pelleted by centrifugation at 7,000 rpm for 30 minutes and 4° C. The supernatant was decanted, save and filtered through a Acrodisc 0.2 μm syringe filter (PN4192, Pall, Ann Arbor, Mich.). K9G9 was purified from the filtered heat treated cell free extract using a GE Healthcare HiLoad 26/60 Superdex 200 gel filtration column (17-1071-01, Buckinghamshire, England). The column was pre-equilibrated with 0.2 CV equilibration with 50 mM HEPES (pH 7.5) 5 mM $MgCl_2$ buffer at a 2.0 mL/min flow rate prior to protein loading, K9G9 and variants were eluted over a 1.5 CV isocratic step consisting of 50 mM HEPES (pH 7.5) 5 mM $MgCl_2$ buffer at a 2.0 mL/min flow rate. Fractions 2.5 mL in volume were collected using a Frac-950 fraction collector (Buckinghamshire, England) in a serpentine pattern, K9G9 and variants all eluted between fractions D5-E5 or D6-E4. Fractions were pooled using a 15 mL Amicon Ultra YM-30 spin filter (UFC903008, Millipore, Billercia, Mass.) and washed with 10 mL 100 mM HEPES (pH 6.8) and 10 mM $MgCl_2$ buffer. Filtrate was discarded and the purified protein was eluted from the membrane using 1 mL buffer containing 100 mM HEPES (pH 6.8) and 10 mM $MgCl_2$.

To determine V_{max} and K_M values for NADH and NADPH, the purified proteins were assayed at various concentrations of NAD(P)H (0 to 1000 μM) coupled with a NAD(P)H regeneration system. Assays were conducted at 30° C. in a buffer containing 100 mM MOPS, pH 6.8, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM (R/S)-acetolactate, 1 mM glucose-6-phosphate, 3 mU/μL glucose-6-phosphate dehydrogenase. The reaction as quenched after ten minutes with three volumes 0.1% formic acid. DHIV concentration was measured using LC-MS. The rate of conversion of S-acetolactate to DHIV was determined by measuring the amount of DHIV produced at a fixed time point. V_{max} and K_m values were calculated by plotting specific activity (U/mg) vs. cofactor concentration and the data were fit to the Michaelis-Menten equation. Measurements of acetolactate Km values (at a fixed concentration of NADH) indicated that the fixed acetolactate concentration employed for the cofactor Km determinations was saturating.

TABLE 23

Kinetic Values for Purified K9G9 Variants as Determined via DHIV Formation Assays

Variant	V_{max} NADPH, U/mg	K_m NADPH, μM	V_{max}/K_m NADPH, L/min * mg	V_{max} NADH, U/mg	K_m NADH, μM	V_{max}/K_m NADH, L/min * mg
K9G9	2.2	24.1	0.091	1.9	78.2	0.024
K9JB4	2.7	249	0.011	3.4	115	0.030
K9JB4P	2	83.2	0.024	2.9	34.1	0.085
K9G3	3.1	113	0.027	2.8	106	0.026
K9G3P	1.8	33.6	0.054	2.1	18.1	0.116
K9JA1	2.6	63.4	0.041	3.4	14	0.243
K9SB2	1.7	44.8	0.038	1.8	11.6	0.155
K9SB2-K90L	2.1	173	0.012	2.4	28.6	0.084
K9SB2-K90M	1.8	245	0.007	2.2	41.3	0.053

Example 19

Isobutanol Production of K9G9 Derivatives with Increased Ratios of K_m NADPH to K_m NADH

The yeast expression plasmids for K9JB4, K9JB4P, K9JG3, K9JG3P, K9JA1, and K9SB2 were made by subcloning of the variant KARI genes from *E. coli* vectors (pBAD.KARI) into pHR81-PIlv5-KARI-K9.G9 at PmeI and SfiI sites. The resultant plasmids together with pHR81-PIlv5-KARI-K9.G9 and pHR81-PIlv5-KARI-K9D3 (SEQ ID NO: 181) were analyzed for isobutanol production and by-product formation in yeast. Yeast pathway strains were made in PNY2259 (MATa ura3Δ::loxP his3Δ pdc6Δ pdc1Δ::P[PDC1]-DHAD|ilvD_.Sm-PDC1t-P[FBA1]-ALS|alsS_.Bs-CYC1t pdc5Δ::P[PDC5]-ADH|sadB_Ax-PDC5t gpd2Δ::loxP fra2Δ::P[PDC1]-ADH|adh_.Hl-ADH1t adh1Δ::UAS(PGK1)P[FBA1]-kivD_Lg(y)-ADH1typrcΔ15Δ::P[PDC5]-ADH|adh_Hl-ADH1t ymr226CA ald6Δ::loxP; Example 22) host by co-transforming the KARI vectors as pathway plasmid #1, and pBP915 (pRS423-P_{FBA1}-DHAD-P_{GPM1}-hADH1; SEQ ID NO: 182) as pathway plasmid #2. The transformed cells were plated on synthetic medium without histidine or uracil (1% ethanol as carbon source). Three transformants were transferred to fresh plates of the same media. The transformants were tested for isobutanol production under anaerobic conditions in serum vials.

Yeast colonies from the transformation on SE-Ura-His plates appeared after 3-5 days. The three colonies from each variant were patched onto fresh SE-Ura-His plates, incubate at 30° C. for 3 days.

Growth Media and Procedure

Two types of media were used during the growth procedure of yeast strains: an aerobic pre-culture media and an anaerobic culture media. All chemicals were obtained from Sigma unless otherwise noted (St. Louis, Mo.)

Aerobic pre-culture media (SE-Ura-His): 6.7 g/L yeast nitrogen base without amino acids (Difco, 291940, Sparks, Md.), 1.4 g/L yeast synthetic drop-out medium supplement without histidine, leucine, tryptophan and uracil, 0.2% ethanol, 0.2% glucose, 0.01% w/v leucine and 0.002% w/v tryptophan.

Anaerobic culture media (SEG-Ura-His): 50 mM MES (pH 5.5, 6.7 g/L yeast nitrogen base without amino acids (Difco, 291940, Sparks, Md.), 1.4 g/L yeast synthetic drop-out medium supplement without histidine, leucine, tryptophan and uracil, 0.1% ethanol, 3% glucose, 0.01% leucine, 0.002% tryptophan, 30 mg/L nicotinic acid, 30 mg/L thiamine and 10 mg/L ergosterol made up in 50/50 v/v Tween/ethanol solution.

The patched cells were inoculated into 25 mL SEG-Ura, His media with 0.2% glucose and 0.2% ethanol, and grown under progressively oxygen-limited conditions with lid closed for approximately 48 hours at 30° C. with shaking, until a target OD_{600} value of approximately 1.5 to 2 was achieved. OD_{600} values were recorded. Cells were pelleted via centrifugation and the supernatant was discarded. Cell pellets were transferred into a Coy Anaerobic Bag (Grass Lake, Mich.) where pellets were resuspended in 1.0 mL anaerobic growth media (SEG-Ura-His). The resuspended cell pellets were used to inoculate 30 mL SEG-Ura-His media in 50 mL serum bottles (Wheaton, 223748, Millville, N.J.) to a target initial OD_{600} value of 0.2. All anaerobic media, serum vials, stoppers and crimps were allowed to degas in the anaerobic bag for at least 24 hours prior to inoculation. Serum bottles were stoppered, crimped and transferred out of the anaerobic bag and grown at 30° C. with shaking at 240 rpm. Anaerobic cultures were grown for 24 to 72 hours with a target OD_{600} value of at least 1.2. Additional anaerobic growth steps used the cells from the previous anaerobic culture step as inoculant. Three transformants were evaluated for each variant.

HPLC Analysis of Yeast Strains with K9G9 KARI Variants

Samples were taken for HPLC analysis and to obtain OD_{600} values at the end of the anaerobic growth period. HPLC analysis was performed using a Waters 2695 separations unit, 2996 photodiode array detector, and 2414 refractive index detector (Waters, Milford, Mass.) with a Shodex Sugar SH-G pre-column and Shodex Sugar SH1011 separations column (Shodex, JM Science, Grand Island, N.Y.). Compounds were separated by isocratic elution at 0.01 N sulfuric acid with a flow rate of 0.5 mL/min. Chromatograms were analyzed using the Waters Empower Pro software.

Molar yields for glycerol, isobutanol and the glycerol/isobutanol ratio were determined. Mean and standard deviations were calculated from triplicate analyses for each variant. Student's t-test was then employed to determine if the difference in the values was statistically significant from the K9D3 control values. For the new variants, the increases in K_M values for NADPH relative to K_M for NADH are expected result reduced NADPH utilization. Results reported in the Table below and in FIG. **9** indicate that the new variants with increased ratios of K_M NADPH to K_M NADH exhibit higher isobutanol to glycerol ratios relative to K9D3 and K9G9. K9SB2 demonstrated a 35% increase in isobutanol titer compared to K9D3.

TABLE 24

K9G9 Variants Kinetic and Isobutanol Data

	Variant	K_m (NADPH)/ K_m (NADH)	Isobutanol/ Glycerol Ratio	Isobutanol Molar Yield	Isobutanol Titer, mM
Experiment 1	K9D3	0.24	1.67 ± 0.02	0.581 ± 0.007	33.9 ± 1.8
	K9JB4	2.2	2.10 ± 0.06	0.603 ± 0.006	35.3 ± 1.3
	K9JG3	1.1	2.07 ± 0.06	0.598 ± 0.004	39.1 ± 1.6
Experiment 2	K9D3	0.24	1.75 ± 0.06	0.586 ± .0100	63.7 ± 0.6
	K9JA1	4.5	2.24 ± 0.04	0.611 ± 0.002	78.2 ± 5.4
	K9SB2	3.9	2.21 ± 0.10	0.608 ± 0.007	77.2 ± 1.4
Experiment 3	K9G9	0.31	2.10 ± 0.03	0.624 ± 0.011	54.2 ± 1.5
	K9JG3P	1.9	2.56 ± 0.08	0.652 ± 0.009	66.3 ± 4.0
	K9JB4P	2.4	2.54 ± 0.07	0.654 ± 0.006	61.5 ± 3.4

Example 20

Construction of K9SB2 Error Prone PCR Library

The K9SB2 error prone PCR library was constructed in a similar manner as the K9G9 library with the following modifications. The mutagenic PCR mixture consisted of 9.5 μl K9SB2 in a pBAD.KARI vector (190 ng/μl), 1.25 μl of primer K9G9_EZ_F1 (100 ng/μl), 1.25 μl of primer K9G9_EZ_R1 (100 ng/μl), 5 μl of 10× Mutazyme H reaction buffer, 1 μl of 40 mM dNTP mix, 1.5 μl of Mutazyme H DNA polymerase, and 30.5 μl of ddH2O. The "EZclone reaction" contained 25 μl of the 2× EZClone enzyme mix, 3 μl of megaprimer (K9SB2 mutagenic PCR product, 190 ng/μl), 2.6 μl of K9SB2 template DNA (19 ng/μl), 3 μl of EZClone solution 1, and 16 μl of ddH_2O. For the Dpn I step, the mixture was incubated for 3 hr at 37° C. Clones were employed for high throughput screening as described in Example 21.

Example 21

Screening for K9SB2 Variants with Further Increased Ratios of Km NADPH to Km NADPH Based on Increased NADH to NADPH Activity Ratios

The K9SB2 library described in Example 20 was screened for variants with reduced NADPH affinity. With the specific objective to increase Km for NADPH relative to Km for NADH, the hits from the screen were partially purified and kinetic analyses were performed to determine V_{max} and K_M parameters with NADH and with NADPH.

High Throughput Screening Assay of K9SB2 Gene Library

Variants were screened using HTS as described in Example 16, with the following exceptions. Assays buffer consisted of 2.4 mM (R/S)-acetolactate, 100 mM HEPES pH 6.8, 10 mM $MgCl_2$, 150 μM NADH or 100 μM NADPH and 12.5 μL 0.5× cell lysate.

The ratio for the measured rate for oxidation of 100 μM NADPH to the measured rate for oxidation of 150 μM NADH was calculated for each variant and positive control well (2 per plate). A variant well was considered to contain an initial hit if the NADH rate was greater than 0.6 OD/hr and the rate ratio (NADPH/NADH) was less than 0.37 (three standard deviations lower than the positive control mean). A total of 218 hits were identified from a pool of 4947 potential variants. These initial hits were consolidated, forming a smaller library for further analysis.

The consolidated initial hit library was grown in biological triplicate and cell free extracts were prepared and assayed as described above. Rate ratios were then calculated for the variants and positive controls as above. Final hits that were selected for detailed kinetic analysis met the following criteria: the NADPH/NADH rate ratio was less than 0.45, the NADH rate was greater than 0.6 OD/hr and at least two out of three biological replicates passed the criteria. 107 variants were identified.

Data were also analyzed to identify variants that had a higher rate of conversion for S-acetolactate to DHIV with the NADH cofactor. The average rate and standard deviation of NADH oxidation was calculated for all the positive controls. A variant was considered a potential hit if the rate of NADH oxidation was at least 3 standard deviations higher than the rate of the positive control (2.524 OD/hr). 68 variants were identified and sequence analysis determined that 17 had at least one amino acid substitution. The substitutions T93A and T93I each appeared twice and variants 2017 B12 and D6 have been selected for further analysis.

DNA sequencing of the 107 variants identified from the secondary HTS screening was accomplished by using TempliPhi™ (GE Healthcare) with the primers pBAD-For (ATGCCATAGCATTTTTATCC; SEQ ID NO: 393) and pBAD-Rev (CTGATTTAATCTGTATCAGGCT; SEQ ID NO: 394). 105 sequences were different from the parent and the amino acid substitutions are listed in the first of the following two tables.

DNA sequencing of the 68 variants identified from the NADH rate screening was accomplished by using TempliPhi™ (GE Healthcare) with primers pBAD-For (ATGCCATAGCATTTTTATCC; SEQ ID NO: 393) and pBAD-Rev (CTGATTTAATCTGTATCAGGCT; SEQ ID NO: 394). 17 sequences were different form wild type and the amino acid substitutions of the 2 substitutions that appeared repeatedly are listed in the second table below.

TABLE 25

K9SB2 Variants' Amino Acid Substitutions

Variant	Seq	Amino Acid Substitutions
K9SB2	427	Y53F, S56A, K57E, S58E, N87P
2011 A2	433	Y53F, G55D, S56A, K57E, S58E, N87P
2011 A3	434	Y53F, S56A, K57E, S58E, N87P, M94I
2011 A5	435	Y53F, S56A, K57E, S58E, M83I, N87P, L185M, E217D
2011 A7	436	Y53F, S56A, K57E, S58E, N87P, D98V
2011 A9	437	Y53F, S56A, K57E, S58E, F67I, N87P
2011 A11	438	Y53F, S56A, K57E, S58E, N87P, M94T, K126E, T273A
2011 B1	439	Y53F, S56A, K57E, S58E, N87P, M94I, A279T
2011 B2	440	Y53F, S56A, K57E, S58E, N87P, Q91L
2011 B3	441	Y53L, S56A, K57E, S58E, N87P
2011 B4	442	Y53F, S56A, K57E, S58E, N87P, P135T
2011 B7	443	Y53F, S56A, K57E, S58E, N87P, L185M
2011 B8	444	Y53F, S56A, K57E, S58E, N87P, C233S, F296Y
2011 B10	445	Y53F, S56A, K57E, S58E, N87P, A303D
2011 C1	446	Y53F, S56A, K57E, S58E, E63K, N87P, G251D, K294R
2011 C3	448	Y53F, S56A, K57E, S58E, A72V, N87P, N102Y, F189I, Y245H
2011 C6	449	Y53F, S56A, K57E, S58E, I84F, N87P
2011 C7	450	E13V, Y53F, S56A, K57E, S58E, M94I, N87P, T141I
2011 C8	451	Y53F, S56A, K57E, S58E, A72V, N87P, N102Y, F189I, Y245H
2011 C9	447	Y53F, S56A, K57E, S58E, N87P, E194D
2011 C10	452	Y53F, A56G, K57E, S58E, K60N, N87P
2011 C12	453	Y53F, S56A, K57E, S58E, I84L, N87P, N97T
2011 D1	454	L39M, Y53F, S56A, K57E, S58E, E68G, N87P
2011 D2	455	Y53F, S56A, K57E, S58E, N87P, M94I, V307I
2011 D3	456	Y53F, S56A, K57E, S58E, F67I, N87P
2011 D4	457	Y53F, S56A, K57E, S58E, N87P, P135L, A202V
2011 D5	458	Y53F, S56A, K57E, S58E, N87P, G164S, G199A
2011 D6	500	Y53F, S56A, K57E, S58E, N87P, S247C
2011 D8	459	Y53F, S56A, K57E, S58E, N87P, N116I
2011 D9	460	Y53F, S56A, K57E, S58E, N87P, K90M
2011 D11	461	Y53F, S56A, K57E, S58E, N87P, M94L, T259I
2011 D12	462	Y53F, S56A, K57E, S58E, M83K, N87P
2011 E3	463	Y53F, S56A, K57E, S58E, N87P, I122V, L297W
2011 E4	464	Y53F, S56A, K57E, S58E, N87P, A112S, Q160R
2011 E8	465	Y53F, S56A, K57E, S58E, N87P, V142I, P320L
2011 E11	466	Y53F, S56A, K57E, S58E, N87P, Q91L
2011 F2	467	Y53F, S56V, K57E, S58E, N87P, A210T
2011 F4	468	Y53F, S56A, K57E, S58E, N87P, F189I
2011 F6	469	Y53F, S56G, K57E, S58E, K60N, N87P
2011 F9	470	A41V, Y53F, S56A, K57E, S58E, N87P, S305P
2011 F10	471	Y53F, S56A, K57E, S58E, L85M, N87P
2011 G1	472	E13V, Y53F, S56A, K57E, S58E, M94I, N87P, T141I
2011 G3	473	H35Q, Y53F, S56A, K57E, S58E, N87P
2011 G4	474	Y53F, S56A, K57E, S58E, I84F, N87P
2011 G8	475	A26T, Y53F, S56A, K57E, S58E, N87P, K90M
2011 G9	476	Y53F, S56T, K57E, S58E, N87P
2011 G10	477	Y53F, S56A, K57E, S58E, I84F, N87P
2011 H1	478	Y53F, S56A, K57E, S58E, N87P, M94I, V156L
2011 H5	479	Y53F, S56A, K57E, S58E, I84L, N87P
2011 H7	480	Y53F, S56T, K57E, S58E, N87P
2011 H9	481	Y53F, G55C, S56A, K57E, S58E, N87P
2012 A2	482	Y53F, S56A, K57E, S58E, R61G, I86V, N87P
2012 A7	483	Y53F, S56A, K57E, S58E, T71S, A76V, N87P
2012 A8	484	Y53F, S56A, K57E, S58E, N87P, M212T
2012 A9	485	H35Q, Y53F, S56A, K57E, S58E, A72T, N87P
2012 A10	486	A36T, Y53F, S56A, K57E, S58E, N87P

TABLE 25-continued

K9SB2 Variants' Amino Acid Substitutions

Variant	Seq	Amino Acid Substitutions
2012 A11	487	Y53F, S56A, K57E, S58E, N87P, S247T
2012 B3	488	Y53F, S56A, K57E, S58E, N87P, P135S
2012 B4	489	Y53F, S56A, K57E, S58E, W59R, N87P, K278E
2012 B5	490	Y53F, S56V, K57E, S58E, N87P, I234V
2012 B6	491	Y30H, Y53F, S56A, K57E, S58E, N87P
2012 B9	492	I50M, Y53F, S56A, K57E, S58E, N87P
2012 C2	493	Y53L, S56A, K57E, S58E, N87P
2012 C3	494	Y53F, S56A, K57E, S58E, N87P, F115V, T191S, V208I, C209W, F292I
2012 C5	495	Y53F, S56A, K57E, S58E, N87P, M94I
2012 C6	496	Y53F, S56A, K57E, S58E, F67L, N87P
2012 C8	497	Y53F, S56A, K57E, S58E, N87P, M94I, M169T
2012 C10	498	Y53F, S56A, K57E, S58E, F67I, N87P, T276I
2012 D1	499	Y53F, S56A, K57E, S58E, I84F, N87P, M132T
2012 D8	501	Y53F, S56A, K57E, S58E, N87P, P135S
2012 D11	502	K8N, Y53F, S56A, K57E, S58E, N87P, K90M, T141I
2012 E5	503	Y53F, S56A, K57E, S58E, I84L, N87P
2012 E9	504	Y53F, S56A, K57E, S58E, N87P, V142I, T191S, C233S
2012 F1	505	Y53F, S56A, K57E, S58E, N87P, H235Y
2012 F2	506	Y53F, S56V, K57E, S58E, N87P, V232D
2012 F3	507	Y53F, S56A, K57E, S58E, N87P, K90M, V142I, T187S
2012 F4	508	Y53F, S56A, K57E, S58E, N87P, M94I, G149D
2012 F7	509	E13V, Y53F, S56A, K57E, S58E, N87P, M94I, T141I
2012 F10	510	Y53F, S56A, K57E, S58E, Q65H, N87P, F189I
2012 F12	511	Y53F, S56V, K57E, S58E, N87P
2012 G3	512	Y53F, S56T, K57E, S58E, N87P, R190S
2012 G4	513	Y53F, S56A, K57E, S58E, N87P, N102S, V142I
2012 G5	514	Y53F, S56A, K57E, S58E, I84L, N87P
2012 G8	515	Y53F, S56A, K57E, S58E, K77N, N87P, A92V
2012 G9	516	Y53F, S56A, K57E, S58E, N87P, M94I, V307I
2012 G10	517	Y53F, S56A, K57E, S58E, N87P, T195I
2012 G12	518	Y53F, S56A, K57E, S58E, N87P, F309I
2012 H1	519	Y53F, S56A, K57E, S58E, N87P, K90T, A180S
2012 H3	520	Y53F, S56A, K57E, S58E, W59C, N87P
2012 H7	521	Y53F, S56A, K57E, S58E, N87P, M94I, A202T
2012 H9	522	H35N, Y53F, S56A, K57E, S58E, N87P
2012 H11	523	Y53F, S56A, K57E, S58E, A72V, N87P, L211M, I240M
2013 A2	524	Y53F, S56T, K57E, S58E, N87P, Q288H
2013 A4	525	Y53F, S56A, K57E, S58E, L85M, N87P
2013 A5	526	L52S, Y53F, S56A, K57E, S58E, N87P
2013 B2	527	A36T, Y53F, S56A, K57E, S58E, N87P, V203I
2013 B5	528	Y53F, S56A, K57E, S58E, N87P, P135T
2013 B7	529	I9M, Y53F, S56A, K57E, S58E, N87P, K90E
2013 B8	530	Y53F, G55C, S56A, K57E, S58E, N87P
2013 B9	531	Y53F, S56V, K57E, S58E, N87P
2013 B11	532	A38V, Y53F, S56A, K57E, S58E, N87P
2013 C1	533	Y53L, S56A, K57E, S58E, N87P, M237I
2013 C6	534	K23M, Y53F, S56A, K57E, S58E, N87P, E194D
2013 C8	535	Y53F, S56A, K57E, S58E, N87P, P135T
2013 C12	536	Y53F, S56A, K57E, S58E, A72V, N87P, T93S, A176V, H235Y
2013 D1	537	Y53F, S56A, K57E, S58E, N87P, M94R, K310M

TABLE 26

K9SB2 Variants' Amino Acid Substitutions

Variant	AA Seq ID NO; Nucleic acid SEQ ID NO	Amino Acid Substitutions
K9SB2	427	Y53F, S56A, K57E, S58E, N87P
2017 B12	639; 640	Y53F, S56A, K57E, S58E, N87P, T93I
2017 D6	641; 642	Y53F, S56A, K57E, S58E, N87P, T93A

Kinetic Analysis of Partially Purified K9SB2 Variant Proteins

E. coli strain Bw25113 (ΔilvC), as described in U.S. Pat. No. 8,129,162, was used to express the 107 variants from the secondary HTS screening and positive control K9SB2. Clones from archive plates were inoculated into the 96-deep well plates. Each well contained 3.0 μl of cells from thawed archive plates, 200 μl of the LB medium containing 100 μg/ml ampicillin and 0.02% (w/v) arabinose as the inducer. Cells were the grown overnight at 37° C. with 80% humidity while shaking (900 rpm), harvested by centrifugation (4000 rpm, 7 min at 4° C.) (75004251, Thermo Scientific, Rockford, Ill.) and the cell pellet was stored at −80° C. for later analysis.

Frozen cell pellets in deep-well plates were thawed at room temperature for 30 minutes at the same time. 75 μl of 50% BugBuster (Novagen 71456, Darmstadt, Germany) (0/in water) was added to each and cells were suspended using a plate shaker. The cells suspension in 50% Bug Buster was incubated for 30 minutes at room temperature which was then followed by a 15 minute incubation at 60° C. Cell debris and denatured heat labile proteins were pelleted by centrifugation (4000 rpm, 15 min at 4° C.) (75004251, Thermo Scientific, Rockford, Ill.) and 75 μL of the supernatant was transferred to a flat bottomed 96-well plate (Corning, 3370, Corning, N.Y.) and diluted two-fold with 75 μL 100 mM HEPES (pH 6.8), 100 mM KCl, 10 mM $MgCl_2$.

Total protein was determined by using the Bradford Assay with Coomaisse Plus (Thermo Scientific, #23238, Rockford, Ill.). BSA was employed as the standard. The concentration of protein was measured by determining the absorbance at 595 nm using a Cary 300 spectrophotometer (Agilent Technologies, Wilmington, Del.).

To determine V_{max} and K_M values for NADH and NADPH, the partially purified proteins were assayed at various concentrations of NADH (20, 30, 40, 60, 80, 120, 200 and 300 μM) and NADPH (60, 80, 120, 200, 300 and 400 μM). Assays were conducted at 30° C. in 100 mM HEPES (pH 6.8), 10 mM $MgCl_2$, 100 mM KCl and 4.8 mM R/S-acetolactate. Between 0.005 to 0.015 mg/mL total protein was added to the assay. The rate of conversion of S-acetolactate to DHIV was measured via monitoring the oxidation of NAD(P)H at 340 nm using a Spectramax 384 Plus plate reader (Molecular Devices, Sunnyvale, Calif.). The activity was calculated using the molar extinction coefficient of 6220 $M^{-1}cm^{-1}$. V_{max} and K_M values were calculated by plotting specific activity (U/mg) vs. cofactor concentration and the data were fit to the Michaelis-Menten equation using Kaleidagraph software (Synergy, Reading, Pa.).

TABLE 27

Kinetic Values for Partially Purified K9SB2 Variants as Determined via NAD(P)H Consumption Assays

Variant	V_{max} NADPH, U/mg	K_m NADPH, μM	V_{max}/K_m NADPH, L/min * mg	V_{max} NADH, U/mg	K_m NADH, μM	V_{max}/K_m NADH, L/min * mg
K9SB2	1.79	153	0.012	2.10	87.4	0.024
2011 A2	1.48	897	0.002	1.94	71.2	0.027
2011 A3	1.43	371	0.004	1.33	44.1	0.030

TABLE 27-continued

Kinetic Values for Partially Purified K9SB2 Variants as Determined via NAD(P)H Consumption Assays

Variant	V_{max} NADPH, U/mg	K_m NADPH, μM	V_{max}/K_m NADPH, L/min * mg	V_{max} NADH, U/mg	K_m NADH, μM	V_{max}/K_m NADH, L/min * mg
2011 A5	0.93	109	0.009	0.23	17.4	0.017
2011 A7	2.27	334	0.007	1.70	69.2	0.025
2011 A9	2.09	266	0.008	0.40	n/d	n/a
2011 A11	2.21	294	0.008	1.25	25.8	0.048
2011 B1	1.79	421	0.004	64.5	55171	0.001
2011 B2	0.33	254	0.001	0.02	264	0.007
2011 B3	1.87	505	0.004	0.68	225	0.003
2011 B4	0.36	294	0.001	0.26	171	0.002
2011 B7	n/d	1.03	n/a	0.48	25.5	0.019
2011 B8	0.83	109.5	0.008	0.61	28.7	0.021
2011 B10	0.88	171	0.005	0.46	7.53	0.061
2011 C1	1.06	404	0.003	1.51	191	0.008
2011 C3	1.08	844	0.001	2.53	500	0.005
2011 C6	1.19	388	0.003	1.90	189	0.010
2011 C7	0.71	946	0.001	1.95	457	0.004
2011 C8	1.73	1546	0.001	3.73	750	0.005
2011 C9	1.02	123	0.008	2.29	177	0.013
2011 C10	1.02	656	0.002	3.84	899	0.004
2011 C12	2.74	244	0.011	3.08	99	0.031
2011 D1	4.68	501	0.009	3.54	80.4	0.044
2011 D2	2.34	547	0.004	2.18	77.3	0.028
2011 D3	0.05	306	0.0002	0.05	44.3	0.001
2011 D4	0.47	857	0.001	0.44	91.6	0.005
2011 D5	0.75	550	0.001	0.42	57.1	0.007
2011 D6	0.70	200	0.004	0.52	25.11	0.021
2011 D8	0.04	214	0.0002	0.04	38.9	0.001
2011 D9	0.18	407	0.0004	0.16	50.2	0.003
2011 D11	0.78	185	0.004	0.61	15.0	0.041
2011 D12	0.74	190	0.004	0.80	39.0	0.021
2011 E3	0.77	163	0.005	1.54	128	0.012
2011 E4	1.59	270	0.006	3.78	234	0.016
2011 E8	0.91	435	0.002	2.16	252	0.009
2011 E11	5.56	6466	0.001	3.06	511	0.006
2011 F2	0.39	692	0.001	1.79	136	0.013
2011 F4	2.07	242	0.009	2.00	68.3	0.029
2011 F6	1.19	946	0.001	1.35	231	0.006
2011 F9	0.57	269	0.002	0.45	27.1	0.017
2011 F10	1.43	390	0.004	1.55	79.8	0.019
2011 G1	1.31	1533	0.001	0.50	40.1	0.013
2011 G3	0.61	1003	0.001	1.00	283	0.004
2011 G4	1.00	316	0.003	2.13	174	0.012
2011 G8	0.90	482	0.002	1.22	106	0.012
2011 G9	0.30	530	0.001	3.48	549	0.006
2011 G10	0.93	358	0.003	2.13	238	0.009
2011 H1	0.96	218	0.004	1.57	114	0.014
2011 H5	1.17	81.2	0.014	1.85	86.5	0.022
2011 H7	0.20	435	0.001	1.25	173	0.007
2011 H9	1.30	741	0.002	1.55	177	0.009
2012 A2	1.71	264	0.007	1.58	55.4	0.029
2012 A7	1.98	215	0.009	1.87	67.3	0.028
2012 A8	1.19	91.8	0.013	1.24	22.7	0.055
2012 A9	0.44	481	0.001	0.34	38.1	0.009
2012 A10	1.21	340	0.004	1.31	66.7	0.020
2012 A11	1.99	342	0.006	1.37	35.9	0.038
2012 B3	0.88	1214	0.001	0.42	63.8	0.007
2012 B4	4.34	1593	0.003	1.66	95.1	0.018
2012 B5	4.88	7389	0.001	1.19	85.2	0.014
2012 B6	3.72	428	0.009	2.23	51.3	0.044
2012 B9	1.17	523	0.002	0.87	63.7	0.014
2012 C2	4.43	923	0.005	3.16	189	0.017
2012 C3	1.40	203	0.007	1.80	68.9	0.026
2012 C5	1.73	348	0.005	3.66	268	0.014
2012 C6	2.18	234	0.009	2.90	103	0.028
2012 C8	1.53	394	0.004	2.63	194	0.014
2012 C10	1.12	286	0.004	1.41	78.9	0.018
2012 D1	0.72	599	0.001	0.58	64.5	0.009
2012 D8	1.17	1528	0.001	0.47	73.6	0.006
2012 D11	0.43	334	0.001	0.52	27.7	0.019
2012 E5	2.13	257	0.008	4.77	313	0.015
2012 E9	1.07	1326	0.001	n/d	n/d	n/a
2012 F1	1.70	272	0.006	1.48	62.5	0.024
2012 F2	0.39	925	0.0004	0.79	97.8	0.008
2012 F3	1.40	2213	0.0006	0.94	142	0.007

TABLE 27-continued

Kinetic Values for Partially Purified K9SB2 Variants as Determined via NAD(P)H Consumption Assays

Variant	V_{max} NADPH, U/mg	K_m NADPH, μM	V_{max}/K_m NADPH, L/min * mg	V_{max} NADH, U/mg	K_m NADH, μM	V_{max}/K_m NADH, L/min * mg
2012 F4	2.70	719	0.004	1.50	72.9	0.021
2012 F7	0.86	840	0.001	0.77	117	0.007
2012 F10	1.92	170	0.011	1.75	27.4	0.064
2012 F12	0.90	1582	0.0006	1.75	117	0.015
2012 G3	1.47	1003	0.002	1.07	127	0.008
2012 G4	0.73	615	0.001	0.63	81.6	0.007
2012 G5	1.92	240	0.008	2.00	83.5	0.024
2012 G8	1.17	315	0.004	0.99	58.7	0.017
2012 G9	2.41	717	0.003	1.37	91.5	0.015
2012 G10	1.06	400	0.003	0.71	39.8	0.018
2012 G12	1.58	147	0.011	2.00	70.0	0.029
2012 H1	1.49	195	0.008	1.74	68.9	0.025
2012 H3	14.98	7389	0.002	1.45	99.0	0.015
2012 H7	1.14	246	0.005	1.30	76.2	0.017
2012 H9	0.37	210	0.002	n/d	n/d	n/a
2012 H11	0.65	162	0.004	0.62	32.0	0.019
2013 A2	0.58	285	0.002	0.64	71.0	0.009
2013 A4	0.63	188	0.003	0.86	81.5	0.011
2013 A5	0.61	886	0.001	0.88	210	0.004
2013 B2	0.62	282	0.002	0.71	70.4	0.010
2013 B5	6.68	7389	0.001	0.083	150	0.006
2013 B7	1.22	433	0.003	1.12	79.0	0.014
2013 B8	0.42	90.7	0.005	1.27	191	0.007
2013 B9	5.31	13970	0.0004	1.38	217	0.006
2013 B11	0.48	212	0.002	0.60	63.2	0.010
2013 C1	0.49	149	0.003	0.68	64.8	0.0105
2013 C6	0.54	163	0.003	0.36	24.4	0.015
2013 C8	2.87	3752	0.001	0.70	188	0.004
2013 C12	0.75	495	0.002	0.79	115	0.007
2013 D1	1.31	1608	0.001	0.87	188	0.005

Example 22

Construction of Strain PNY2259

The purpose of this example is to describe the assembly of the constructs used to replace the chromosomal copy of kivD_Ll(y) in PNY2238 at the adh1Δ locus with kivD_Lg(y).

The deletion/integration was created by homologous recombination with PCR products containing regions of homology upstream and downstream of the target region and the URA3 gene for selection of transformants. The URA3 gene was removed by homologous recombination to create a starless deletion/integration. The plasmid to integrate kivD_Lg(y) was derived from a plasmid constructed to integrate UAS(PGK1)P[FBA1]-kivD_Ll(y) into the ADH1 locus of *Saccharomyces cerevisiae*. Construction of the plasmid used to integrate UAS(PGK1)P[FBA1]-kivD_Ll(y) into the ADH1 locus is described below. The plasmids were constructed in pUC19-URA3MCS.

Construction of the ADH1 Deletion/UAS(PGK1)P[FBA1]-kivD_Ll(y) Integration Plasmid

The kivD coding region from *Lactococcus lactis* codon optimized for expression in *Saccharomyces cerevisiae*, kivD_Ll(y), was amplified using pLH468 (SEQ ID NO: 139) as template with primer oBP562 (SEQ ID NO: 197), containing a PmeI restriction site, and primer oBP563 (SEQ ID NO: 198), containing a 5' tail with homology to the 5' end of ADH1 Fragment B. ADH1 Fragment B was amplified from *Saccharomyces cerevisiae* CEN.PK 113-70 genomic DNA with primer oBP564 (SEQ ID NO: 199), containing a 5° tail with homology to the 3' end of kivD_Ll(y), and primer oBP565 (SEQ ID NO: 200), containing a FseI restriction site. PCR products were purified with a PCR Purification kit (Qiagen; Valencia, Calif.). kivD_Ll(y)-ADH1 Fragment B was created by overlapping PCR by mixing the kivD_Ll(y) and ADH1 Fragment B PCR products and amplifying with primers oBP562 (SEQ ID NO: 197) and oBP565 (SEQ ID NO: 200). The resulting PCR product was digested with PmeI and FseI and ligated with T4 DNA ligase into the corresponding sites of pUC19-URA3MCS after digestion with the appropriate enzymes. ADH1 Fragment A was amplified from genomic DNA with primer oBP505 (SEQ ID NO: 201), containing a SacI restriction site, and primer oBP506 (SEQ ID NO: 202), containing an AscI restriction site. The ADH1 Fragment A PCR product was digested with SacI and AscI and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing kivD_Ll(y)-ADH1 Fragment B. ADH1 Fragment C was amplified from genomic DNA with primer oBP507 (SEQ ID NO: 203), containing a Pad restriction site, and primer oBP508 (SEQ ID NO: 204), containing a SalI restriction site. The ADH1 Fragment C PCR product was digested with Pad and SalI and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing ADH1 Fragment A-kivD_Ll(y)-ADH1 Fragment B. The hybrid promoter UAS(PGK1)-P_{FBA1} (SEQ ID NO: 406) was amplified

from vector pRS316-UAS(PGK1)-P_{FBA1}-GUS with primer oBP674 (SEQ ID NO: 205), containing an AscI restriction site, and primer oBP675 (SEQ ID NO: 206), containing a PmeI restriction site. The UAS(PGK1)-P_{FBA1} PCR product was digested with AscI and PmeI and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing kivD_Ll(y)-ADH1 Fragments ABC to generate pBP1181.

Construction of pBP1716 and pBP1719

kivD_Ll(y) was removed from the ADH1 deletion/UAS (PGK1)P[FBA1]-kivD_Ll(y) integration plasmid pBP1181. The plasmid was digested with PmeI and FseI and the large DNA fragment was purified on an agarose gel followed by a gel extraction kit (Qiagen). ADH1 fragment B was amplified from pBP1181 with primer oBP821 (SEQ ID NO: 407), containing a PmeI restriction site, and primer oBP484 (SEQ ID NO: 408), containing a FseI restriction site. The ADH1 fragment B PCR product was digested with PmeI and FseI and ligated with T4 DNA ligase into the corresponding sites of the gel purified large DNA fragment. A PCR fragment corresponding to the 3' 500 bp of kivD_Ll(y) was cloned into the resulting vector for the targeted deletion of kivD_Ll(y) in PNY1528. The fragment was amplified from pBP1181 with primers oBP822 (SEQ ID NO: 409), containing a NotI restriction site, and oBP823 (SEQ ID NO: 410), containing a Pad restriction site. The fragment was digested with NotI and PacI and ligated with T4 DNA ligase into the corresponding sites downstream of URA3 in the above plasmid with the kivD_Ll(y) deletion after digestion with the appropriate restriction enzymes. The resulting plasmid was designated pBP1716.

The kivD coding region from *Listeria* grayi codon optimized for expression in *Saccharomyces cerevisiae* (SEQ ID NO: 411), kivD_Lg(y), was synthesized by DNA2.0 (Menlo Park, Calif.). kivD_Lg(y) was amplified with primers oBP828 (SEQ ID NO: 412), containing a PmeI restriction site, and oBP829 (SEQ ID NO: 413) containing a PmeI restriction site. The resulting PCR product was digested with PmeI and ligated with T4 DNA ligase into the corresponding site in pBP1716 after digestion with the appropriate enzyme. The orientation of the cloned gene was checked by PCR with primers FBAp-F (SEQ ID NO: 414) and oBP829 (SEQ ID NO: 413). An isolate with kivD_Lg(y) in the correct orientation was designated pBP1719.

Construction of Strain PNY2259

The kivD_Ll(y) deletion/kivD_Lg(y) integration cassette was amplified from pBP1719 with primers oBP505 (SEQ ID NO: 201) and oBP823 (SEC) ID NO: 410). Competent cells of the PNY2238 were made and transformed with the PCR product using a Frozen-EZ Yeast Transformation H kit (Zymo Research; Orange, Calif.). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 1% ethanol at 30 C. Transformant strains were screened by PCR (JumpStart™ REDTaq (c) ReadyMix™) using primers Ura3-end F (SEQ ID NO: 222) and HY-50 (SEQ ID NO: 415). Transformants were grown in YPE (1% ethanol) and plated on synthetic complete medium supplemented with 1% EtOH and containing 5-fluoro-orotic acid (0.1%) at 30 C to select for isolates that lost the URA3 marker. The deletion of kivD_Ll(y) and integration of kivD_Lg(y) was confirmed by PCR with primers HY-50 and oBP834 (SEQ ID NO: 416). One correct isolate contained kivD_Lg(y) at the same locus and expressed from the same promoter as kivD_Ll(y) in PNY2238 was designated PNY2259.

Example 23

Construction of Two Site-Saturation Gene Libraries to Identify Variants with Cofactor Preference to NADH

In Example 4, primers having the degeneracy codon NNK were used (N represents all 4 nucleotides A, C G and T while K stands for G and T). In this Example, primer mixtures containing primers encoding each individual amino acid change of A, C, D, E, F, G, H, I, L, M, N, P, O, V, W, or Y for positions 53, 56 and 58 of K9 KARI were employed and substitutions to S, T, K, and R were excluded as non-preferred for these positions. The size of the saturation library targeting the three NADPH phosphate binding sites (53, 56 and 58) of K9 KARI is 4,096 (as compared to 32*32*32 or 32,768 variants using NNK degeneracy code primers as in Example 4).

One library construction method started from position 58. Primer mixtures were first made by mixing all the primers targeting the same positions (for example, the primer mixture, K9_53f, was made by mixing equal mole of all 16 forward primers targeting position 53 (listed in the table below). Similarly, K9_56f and K9_58f were prepared. The shared reverse primer is K9_191G_112210r (SEQ ID NO: 174): GGTTTCAGTTTCGCCTCTGAAGGTAGTTTC (called SR in this example). The mutation at position 58 was first introduced into AS6F1 through PCR. The mutagenesis procedure is similar to the one described in Example 4. In brief, K9_58f and SR were phosphorylated. The phosphorylated primers were then directly used to introduce mutation at position 58 into AS6F1 using USB Change_It kit (USB Corporation, Cleveland, Ohio, #78480). The template was removed with Dpn I. The cleaned up PCR product (Zymo DNA Clean & Concentrator-5; Zymo Research Corporation, Irvine, Calif., Cat #D4003) was transformed into KOBW-3a cells. After overnight growth on LB agar plates at a 37° C. incubator, all cells were collected and DNA was extracted using the Qiaprep Spin miniprep kit (Qiagen Inc. Valencia, Calif., Cat #27106)

The extracted DNA was then used as templates to introduce mutation at position 56 using K9_56f and SR same as the mutagenesis for position 58. At last the mutation at position 53 was similarly introduced. After mutations at all three positions (53, 56 and 58) were introduced into AS6F1, the new library was screened same as the one described in example 4 and some selected mutants are listed in the table below.

The other method began with the position 53. The primer mixtures K9_56r and K9_58r were similarly prepared using primers listed in the table below). The shared forward primer is pBAD_266f: CTCTCTACTGTTTCTCCATACCCG (SEQ ID NO: 634; called SF in this example). The mutation at position 53 was first introduced into AS6F1 through PCR. The mutagenesis procedure is similar to the one described above using AS6F1 as the template and K9_53f and SR as the two PCR primers. The resulted mutated DNA (at position 53) was used as templates and K9_56r and SF were used as the mutagenesis primers to introduced mutation at position 56. At last the mutation at position 58 was similarly introduced using K9_58r and SF. After mutations at all three positions (53, 56 and 58) were introduced into AS6F1, the new library was screened same as above and some selected mutants were listed in the table below.

TABLE 28

forward mutational primers.

Targeted position(s) of K9-KARI	Primers	
53	K9_53F_031411f	GTTATCATCGGATTATTCGAAGGA (SEQ ID NO: 544)
	K9_53L_031411f	GTTATCATCGGATTATTGGAAGGA (SEQ ID NO: 545)
	K9_53Y_031411f	GTTATCATCGGATTATATGAAGGA (SEQ ID NO: 546)
	K9_53C_031411f	GTTATCATCGGATTATGTGAAGGA (SEQ ID NO: 547)
	K9_53W_031411f	GTTATCATCGGATTATGGGAAGGA (SEQ ID NO: 548)
	K9_53P_031411f	GTTATCATCGGATTACCAGAAGGA (SEQ ID NO: 549)
	K9_53H_031411f	GTTATCATCGGATTACATGAAGGA (SEQ ID NO: 550)
	K9_53Q_031411f	GTTATCATCGGATTACAAGAAGGA (SEQ ID NO: 551)
	K9_53I_031411f	GTTATCATCGGATTAATTGAAGGA (SEQ ID NO: 552)
	K9_53M_031411f	GTTATCATCGGATTAATGGAAGGA (SEQ ID NO: 553)
	K9_53N_031411f	GTTATCATCGGATTAAATGAAGGA (SEQ ID NO: 554)
	K9_53V_031411f	GTTATCATCGGATTAGTTGAAGGA (SEQ ID NO: 555)
	K9_53A_031411f	GTTATCATCGGATTAGCTGAAGGA (SEQ ID NO: 556)
	K9_53D_031411f	GTTATCATCGGATTAGATGAAGGA (SEQ ID NO: 557)
	K9_53E_031411f	GTTATCATCGGATTAGAAGAAGGA (SEQ ID NO: 558)
	K9_53G_031411f	GTTATCATCGGATTAGGTGAAGGA (SEQ ID NO: 559)
56	K9_56K_031411f	GGATTACCTGAAGGATTCAAA (SEQ ID NO: 560)
	K9_56K_031411f	GGATTACCTGAAGGATTGAAA (SEQ ID NO: 561)
	K9_56Y_031411f	GGATTACCTGAAGGATATAAA (SEQ ID NO: 562)
	K9_56C_031411f	GGATTACCTGAAGGATGTAAA (SEQ ID NO: 563)
	K9_56W_031411f	GGATTACCTGAAGGATGGAAA (SEQ ID NO: 564)
	K9_56P_031411f	GGATTACCTGAAGGACCAAAA (SEQ ID NO: 565)
	K9_56H_031411f	GGATTACCTGAAGGACATAAA (SEQ ID NO: 566)
	K9_56Q_031411f	GGATTACCTGAAGGACAAAAA (SEQ ID NO: 567)
	K9_56I_031411f	GGATTACCTGAAGGAATTAAA (SEQ ID NO: 568)
	K9_56M_031411f	GGATTACCTGAAGGAATGAAA (SEQ ID NO: 569)
	K9_56N_031411f	GGATTACCTGAAGGAAATAAA (SEQ ID NO: 570)
	K9_56V_031411f	GGATTACCTGAAGGAGTTAAA (SEQ ID NO: 571)
	K9_56A_031411f	GGATTACCTGAAGGAGCTAAA (SEQ ID NO: 572)
	K9_56D_031411f	GGATTACCTGAAGGAGATAAA (SEQ ID NO: 573)
	K9_56E_031411f	GGATTACCTGAAGGAGAAAAA (SEQ ID NO: 574)
	K9_56G_031411f	GGATTACCTGAAGGAGGTAAA (SEQ ID NO: 575)
58	K9_58F_051611f	GATTACCTGAAGGATTTAAATTCTGGAAGAGAGC (SEQ ID NO: 576)
	K9_58L_051611f	GATTACCTGAAGGATTTAAATTGTGGAAGAGAGC (SEQ ID NO: 517)
	K9_58Y_051611f	GATTACCTGAAGGATTTAAATATTGGAAGAGAGC (SEQ ID NO: 578)
	K9_58C_051611f	GATTACCTGAAGGATTTAAATGTTGGAAGAGAGC (SEQ ID NO: 579)
	K9_58W_051611f	GATTACCTGAAGGATTTAAATGGTGGAAGAGAGC (SEQ ID NO: 580)
	K9_58P_051611f	GATTACCTGAAGGATTTAAACCATGGAAGAGAGC (SEQ ID NO: 581)
	K9_58H_051611f	GATTACCTGAAGGATTTAAACATTGGAAGAGAGC (SEQ ID NO: 582)
	K9_58Q_051611f	GATTACCTGAAGGATTTAAACAATGGAAGAGAGC (SEQ ID NO: 583)
	K9_58I_051611f	GATTACCTGAAGGATTTAAAATTTGGAAGAGAGC (SEQ ID NO: 584)
	K9_58M_051611f	GATTACCTGAAGGATTTAAAATGTGGAAGAGAGC (SEQ ID NO: 585)
	K9_58N_051611f	GATTACCTGAAGGATTTAAAAATTGGAAGAGAGC (SEQ ID NO: 586)
	K9_58V_051611f	GATTACCTGAAGGATTTAAAGTTTGGAAGAGAGC (SEQ ID NO: 587)
	K9_58A_051611f	GATTACCTGAAGGATTTAAAGCTTGGAAGAGAGC (SEQ ID NO: 588)
	K9_58D_051611f	GATTACCTGAAGGATTTAAAGATTGGAAGAGAGC (SEQ ID NO: 589)
	K9_58E_051611f	GATTACCTGAAGGATTTAAAGAATGGAAGAGAGC (SEQ ID NO: 590)
	K9_58G_051611f	GATTACCTGAAGGATTTAAAGGTTGGAAGAGAGC (SEQ ID NO: 591)

TABLE 29

forward mutational primers.

Targeted positior(s) of Kg-KARI	Primers	
56	K9_56F_071211r	GCTCTCTTCCATGGTTTGAATCCTTC (SEQ ID NO: 592)
	K9_56L_071211r	GCTCTCTTCCATGGTTTCAATCCTTC (SEQ ID NO: 593)
	K9_56Y_071211r	GCTCTCTTCCATGGTTTATATCCTTC (SEQ ID NO: 594)
	K9_56C_071211r	GCTCTCTTCCATGGTTTACATCCTTC (SEQ ID NO: 595)
	K9_56W_071211r	GCTCTCTTCCATGGTTTCCATCCTTC (SEQ ID NO: 596)
	K9_56P_071211r	GCTCTCTTCCATGGTTTTGGTCCTTC (SEQ ID NO: 597)
	K9_56H_071211r	GCTCTCTTCCATGGTTTATGTCCTTC (SEQ ID NO: 598)
	K9_56Q_071211r	GCTCTCTTCCATGGTTTTTGTCCTTC (SEQ ID NO: 599)
	K9_56I_071211r	GCTCTCTTCCATGGTTTAATTCCTTC (SEQ ID NO: 600)
	K9_56M_071211r	GCTCTCTTCCATGGTTTCATTCCTTC (SEQ ID NO: 601)
	K9_56N_071211r	GCTCTCTTCCATGGTTTATTTCCTTC (SEQ ID NO: 602)

TABLE 29 -continued

forward mutational primers.

Targeted positior(s) of Kg-KARI	Primers	
	K9_56V_071211r	GCTCTCTTCCATGGTTTAACTCCTTC (SEQ ID NO: 603)
	K9_56A_071211r	GCTCTCTTCCATGGTTTAGCTCCTTC (SEQ ID NO: 604)
	K9_56D_071211r	GCTCTCTTCCATGGTTTATCTCCTTC (SEQ ID NO: 605)
	K9_56E_071211r	GCTCTCTTCCATGGTTTTTCTCCTTC (SEQ ID NO: 606)
	K9_56G_071211r	GCTCTCTTCCATGGTTTACCTCCTTC (SEQ ID NO: 607)
58	K9_58F_071211r	GTTCTTCTGCTCTCTTCCAGAATTT (SEQ ID NO: 608)
	K9_58L_071211r	GTTCTTCTGCTCTCTTCCACAATTT (SEQ ID NO: 609)
	K9_58Y_071211r	GTTCTTCTGCTCTCTTCCAATATTT (SEQ ID NO: 610)
	K9_58C_071211r	GTTCTTCTGCTCTCTTCCAACATTT (SEQ ID NO: 611)
	K9_58W_071211r	GTTCTTCTGCTCTCTTCCACCATTT (SEQ ID NO: 612)
	K9_58P_071211r	GTTCTTCTGCTCTCTTCCATGGTTT (SEQ ID NO: 613)
	K9_58H_071211r	GTTCTTCTGCTCTCTTCCAATGTTT (SEQ ID NO: 614)
	K9_58Q_071211r	GTTCTTCTGCTCTCTTCCATTGTTT (SEQ ID NO: 615)
	K9_58I_071211r	GTTCTTCTGCTCTCTTCCAAATTTT (SEQ ID NO: 616)
	K9_58M_071211r	GTTCTTCTGCTCTCTTCCACATTTT (SEQ ID NO: 617)
	K9_58N_071211r	GTTCTTCTGCTCTCTTCCAATTTTT (SEQ ID NO: 618)
	K9_58V_071211r	GTTCTTCTGCTCTCTTCCAAACTTT (SEQ ID NO: 619)
	K9_58A_071211r	GTTCTTCTGCTCTCTTCCAAGCTTT (SEQ ID NO: 620)
	K9_58D_071211r	GTTCTTCTGCTCTCTTCCAATCTTT (SEQ ID NO: 621)
	K9_585_071211r	GTTCTTCTGCTCTCTTCCATTCTTT (SEQ ID NO: 622)
	K9_58G_071211r	GTTCTTCTGCTCTCTTCCKACCTTT (SEQ ID NO: 623)

TABLE 30

List of some mutants with their measured K_M values

Mutant	SEQ ID NO: (nucleic acid, amino acid)	Mutations	K_M (μM) (NADH)	K_M (μM) (NADPH)
K9 Wt	26, 27	—	326	0.2
BI7D12	625, 624	Y53Q, S56V, S58D, I86V, N87P, T131M, T191G	39	196
BI10F1	627, 626	Y53E, S56V, S58D, I86V, N87P, T131M, T191G	74	573
BJ6G6	629, 628	Y53P, S56D, S58Q, I86V, N87P, T131M, T191G	298	672
BJ7D6	631, 630	Y53P, S56V, S58E, I86V, N87P, T131M, T191G	37	236
BJ7F7	633, 632	Y53A, S56D, S58Q, I86V, N87P, T131M, T191G	269	762

Example 24

Construction of an ald6Δ Strain and Isobutanol-Producing Derivatives

A 5.3 kb (BglII/EcoRV) DNA fragment from pRS426::GPD-xpk1+ADH-eutD (SEQ ID NO:383) containing expression cassettes for xpk1 and eutD genes from *Lactobacillus plantarum* was added between the ALD6 flanking sequences, at the SnaBI site of the pUC19::ald6D::loxP-URA3-loxP vector described in Example 9, above. The ligation reaction was transformed into *E. coli* Stb13 cells, which were incubated on LB Amp plates to select for transformants. Insertion of the xpk1-eutD cassette was confirmed by PCR (primers). A positive clone (pUC19::Δald6::URA3::xpkS) was obtained.

The vector described above was linearized with AhdI and transformed into PNY1507 (described herein) cells prepared with the Zymo Research Frozen-EZ Yeast Transformation Kit (Cat. No. T2001) with a modification to manufacturer's protocol that included an additional outgrowth incubation of 2.5 hrs. in 2.0 mL YPE (yeast extract, peptone with 1% ethanol) medium. Transformants were obtained by plating on synthetic complete medium minus uracil that provided 1% ethanol as the carbon source. Patched transformants were screened by PCR to confirm the deletion/integration, using primers N1090 and N1213 (SEQ ID NOs: 779 and 242). A plasmid carrying Cre recombinase (pRS423::GAL1p-Cre; SEQ ID No. 271) was transformed into the strain using histidine marker selection. Transformants were passaged on YPE supplemented with 0.5% galactose. Colonies were screened for resistance to 5-FOA (loss of URA3 marker) and for histidine auxotrophy (loss of the Cre plasmid). Proper removal of the URA3 gene via the flanking loxP sites was confirmed by PCR with primers N1212 and N1214 (SEQ ID NOs: 241 and 281). Finally, the alsS integration plasmid (SEQ ID NO:780) was transformed into this strain using the included geneticin selection marker. Integrants were confirmed using primers N160SeqF5 and oBP512 (SEQ ID NO: 388 and 337).

Plasmids pYZ090ΔalsS and pBP915 (SEQ ID NOs: 371 and 182) were transformed into the strain by lithium acetate transformation (Protocol #2 in "Methods in Yeast Genetics" 2005. Amberg, Burke and Strathern). Transformants were selected by plating on synthetic complete minus histidine and uracil with ethanol as the carbon source. Transformants were patched and then repatched onto synthetic complete minus histidine and uracil with 2% glucose and 0.05% ethanol. Six clones were evaluated for growth and isobutanol production. One of these has been designated PNY2216.

Example 25

YMR226C Deletion from *S. cerevisiae* Strain PNY2211

Construction of PNY2248

The gene YMR226C was deleted from *S. cerevisiae* strain PNY2211 (described in Example 9) by homologous recom-

bination using a PCR amplified linear KanMX4-based deletion cassette available in *S. cerevisiae* strain BY4743 ymr226CΔ::KanMX4 (ATCC 4020812). Forward and reverse PCR primers N1237 (SEQ ID NO:784) and N1238 (SEQ ID NO:785), amplified a 2,051 bp ymr226CΔ::KanMX4 deletion cassette from chromosome XIII. The PCR product contained upstream and downstream sequences of 253 and 217 bp, respectively, flanking the ymr226CΔ::KanMX4 deletion cassette, that are 100% homologous to the sequences flanking the native YMR226C locus in strain PNY2211. Recombination and genetic exchange occur at the flanking homologous sequences effectively deleting the YMR226C gene and integrating the ymr226CΔ::KanMX4 deletion cassette.

Approximately 2.0 μg of the PCR amplified product was transformed into strain PNY2211 made competent using the lithium-acetate method previously described in *Methods in Yeast Genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp. 201-202 (2005)), and the transformation mix was plated on YPE plus geneticin (50 μg/mL) and incubated at 30° C. for selection of cells with an integrated ymr226CΔ::KanMX4 cassette. Transformants were screened for ymr226cΔ::KanMX4 by PCR, with a 5° outward facing KanMX4 deletion cassette-specific internal primer N1240 (SEQ ID NO:786) paired with a flanking inward facing chromosome-specific primer N1239 (SEQ ID NO:243) and a 3' outward-facing KanMX4 deletion cassette-specific primer N1241 (SEQ ID NO:787) paired with a flanking inward-facing chromosome-specific primer N1242 (SEQ ID NO:244). Positive PNY2211 ymr226C1::KanMX4 clones were obtained, one of which was designated PNY2248.

Example 26

Production of Isobutanol with Decreased DHMB Yield in YMR226c Knock-Out

PNY2211 ymr226CΔ::KanMX4 transformants and a non-deletion control (PNY2211 with native YMR226C) were tested for butanol production in glucose medium by first introducing the isobutanol pathway-containing plasmids pYZ090ΔalsS (SEQ ID NO:371) and pBP915 (SEQ ID NO:182) simultaneously by the Quick and Dirty lithium acetate transformation method described in *Methods in Yeast Genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2005)). Plasmid selection was based on histidine and uracil auxotrophy on selection plates containing ethanol (synthetic complete medium with 1.0% ethanol-his-ura). After three to five days, several transformants showing the most robust growth were adapted to glucose medium by patching onto SD 2.0% glucose+0.05% ethanol-his-ura and incubated 48 to 72 hours at 300° C. Three streaks showing the most robust growth were used to inoculate a 10 mL seed culture in SD 0.2% glucose+0.2% ethanol-his-ura in 125 mL vented flasks and grown at 30° C., 250 rpm for approximately 24 hours. Cells were then subcultured into synthetic complete medium with 2% glucose+0.05% ethanol-his-ura in 125 ml tightly-capped flasks and incubated 48 hours at 30° C. Culture supernatants collected after inoculation and after 48 hours incubation were analyzed by HPLC to determine production of isobutanol and by LC/MS to quantify DHMB. Controls strains were observed to produce DHMB at a molar yield of 0.03 to 0.07 mole per mole glucose. A peak corresponding to DHMB was not observed in culture supernatants of the ymr226cΔ strains, one of which was designated PNY2249.

Example 27

Identification of Genes that Encode Acetolactate Reductase (ALR) Activity Enzymes Using Yeast Knockout Library

From a knockout ("KO") collection of >6000 yeast strains derived from the strain BY4743, available from Open Biosystems® (a division of Thermo Fisher Scientific, Waltham, Mass.), 95 candidate dehydrogenase gene knockout strains were chosen. Starter cultures of knockout strains were grown in 96-well deepwell plates (Costar 3960, Corning Inc., Corning N.Y., or similar) on rich medium YPD, and subcultured at a starting OD 600 nm of ~0.3 in medium containing 0.67% Yeast Nitrogen Base, 0.1% casamino acids, 2% glucose, and 0.1 M K^+-MES, pH 5.5. Samples were taken over a 5-day period for DHMB and DHIV measurements. DHIV and the two isomers of DHMB were separated and quantified by liquid chromatography-mass spectrometry ("LC/MS") on a Waters (Milford, Mass.) AcquityTQD system, using an Atlantis 13 (part #186003539) column. The column was maintained at 30° C., and the flow rare was 0.5 ml/min. The A mobile phase was 0.1% formic acid in water, and the B mobile phase was 0.1% formic acid in acetonitrile. Each run consisted of 1 min at 99% A, a linear gradient over 1 min to 25% B, followed by 1 min at 99% A. The column effluent was monitored for peaks at m/z=133 (negative ESI), with cone voltage 32.5V, by Waters ACQ_TQD (s/n QBA688) mass spec detector. The so-called "fast DHMB" typically emerged at 1.10 min, followed by DHIV at 1.2 min, and "slow" DHMB emerged at 1.75 min. Baseline separation was obtained and peak areas for DHIV were converted to μM DHIV concentrations by reference to analyses of standards solutions made from a 1M aqueous stock. These measurements showed that most of the changes in DHMB levels occurred in the first 48-60 hours, so a single sample was collected at about that time in subsequent experiments. In this experiment, fast DHMB was found at much higher levels than slow DHMB, which was not always detectable. The ratio of DHIV to fast DHMB in most cultures was ~3, but a strain lacking the YMR226C gene consistently showed very low levels of fast DHMB, and normal DHIV, so that the DHIV/fast DHMB ratio was about 100. This suggested that YMR226Cp is the major ALR in this background.

To confirm that YMR226Cp is the major ALR in this background, the in vitro levels of ALR and KARI were tested in the ymr226C deletion strain (American Type Culture Collection (ATCC), Manassas Va., ATCC #4020812) and its parent, BY4743 (ATCC #201390; American Type Culture Collection, Manassas Va.). Fifty ml tubes containing 6 ml YPD were inoculated from YPD agar plates and allowed to grow overnight (30° C., 250 rpm). The cells were pelleted, washed once in water, and resuspended in 1 ml yeast cytoplasm buffer (Van Eunen et al. *FEBS Journal* 277: 749-760 (2010)) containing a yeast protease inhibitor cocktail (Roche, Basel, Switzerland, Cat #11836170001, used as directed by the vendor, 1 tablet per 10 mls of buffer). Toluene (0.02 ml, Fisher Scientific, Fair Lawn N.J.) was added, and the tubes were shaken at top speed for 10 min on a Vortex Genie 2 shaker (Scientific Industries, Bohemia N.Y., Model G-560) for permeabilization. The tubes were placed in a water bath at 30° C., and substrates were added to the following final concentrations: (S)-acetolactate (made enzymatically as described below in Example 29) to 9.4 mM, NADPH (Sigma-Aldrich, St. Louis Mo.) 0.2 mM plus a NAD(P)H-regeneration system consisting of 10 mM glucose-6-phosphate and 2.5 U/ml *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (Sigma,

St. Louis, Mo., Cat # G8404). At timed intervals, aliquots (0.15 ml) were added to 0.15 ml aliquots of 2% formic acid to stop the reaction. The samples were then analyzed for DHIV and both isomers of DHMB by LC/MS as described above; only fast DHMB and DHIV were observed. The specific activities of the two enzymes in the two strains are shown in Table 31.

TABLE 31

KARI and ALR Enzyme Activities

Strain	KARI	ALR
BY4743	1.7 mU/mg protein	20 mU/mg
YMR226C deletion strain	2.2 mU/mg protein	0.1 mU/mg

The data suggests that the YMR226C gene product accounted for >99% of the ALR activity.

Example 28

Identification of Genes that Encode Acetolactase Reductase (ALR) Activity Enzymes Using Yeast Overexpression Library

From a "Yeast ORF" collection of >5000 transformants of Y258 each with a plasmid carrying a known yeast gene plus a C-terminal tag, under the control of an inducible promoter (Open Biosystems®, a division of Thermo Fisher Scientific, Waltham, Mass.), ninety-six strains with plasmids containing genes associated with dehydrogenase activity were grown in 96-well format by adaptation of the growth and induction protocol recommended by the vendor (Open Biosystems®). The cells were pelleted and permeabilized with toluene as described above, and a concentrated substrate mix was added to give final concentrations as in Example 27. Timed samples were taken and analyzed for DHIV and both isomers of DHMB. The ratios of the ALR/KARI were calculated and compared. Strains with elevated ratios were candidates for overproduction of ALR activities. When the data for relative rates of fast DHMB and DHIV formation were displayed in a Minitab® (Microsoft Inc., Redmond, Wash.) boxplot, half the ratios fell between ~9-13, and most of the rest fell within 3 and 19. The exceptions identified as outliers included YER081W, YIL074C, YMR226C, YBR006W, and YOR375c, for which ratios of ALR/KARI fell between 22 and 40. In a similar analysis of relative rates of slow DHMB and DHIV formation, half the ratios fell between 9 and 11, but YMR226C, YPL275W, YER081W, AND YOL059W appeared as outliers, with ratios between 13 and 25. Thus, overexpression of YMR226C and YER081W, increased synthesis of both DHMBs. In addition, YIL074C, YBR006W, and YOR375c increased fast DHMB synthesis, and YPL275W and YOL059W increased slow DHMB synthesis. The genomic DNA sequences (which may include introns) and ORF translation sequences of genes identified in overexpression are provided in Table 6.

Example 29

Inhibition of KARI by DHMB

Enzymatic Production of (S)-Acetolactate

(S)-acetolactate was used as a starting material for DHMB synthesis. (S)-acetolactate was made enzymatically, as follows. An *E. coli* TOP10 strain (Invitrogen, Carlsbad, Calif.) modified to express *Klebsiella* BudB (previously described in U.S. Pat. No. 7,851,188, which is herein incorporated by reference in its entirety; see Example 9 of that patent) under IPTG control was used as a source of enzyme. It was grown in 200-1000 ml culture volumes. For example, 200 ml was grown in Luria Broth (Mediatech, Manassas, Va.) containing 0.1 mg/ml Ampicillin (Sigma, St. Louis, Mo.) in a 0.5 L conical flask, which was shaken at 250 rpm at 37° C. At OD 600 ~0.4, isopropylthiogalactoside (Sigma, St. Louis, Mo.) was added to 0.4 mM, and growth was continued for 2 hours before the cells were collected by centrifugation, yielding ~1 g wet weight cells. Likewise, partial purifications were conducted at scales from ~0.5 to 5 g wet cells. For example, ~0.5 g cells were suspended in 2.5 ml buffer containing 25 mM Na-MES pH 6, broken by sonication at 0° C., and clarified by centrifugation. Crude extract was supplemented with 0.1 mM thiamin pyrophosphate, 10 mM $MgCl_2$, and 1 mM EDTA (all from Sigma, St. Louis, Mo.). Next, 0.07 ml of 10% w/v aqueous streptomycin sulfate (Sigma, St. Louis, Mo.) was added and the sample was heated in a 56° C. water bath for 20 min. It was clarified by centrifugation, and ammonium sulfate was added to 50% of saturation. The mixture was centrifuged, and the pellet was brought up in 0.5 ml 25 mM Na-MES, pH 6.2, and used without further characterization. Acetolactate syntheses were also conducted at various scales. A large preparation was conducted as follows: 5.5 g sodium pyruvate was dissolved in 25 mM Na-MES, pH 6.2, to ~45 ml and supplemented with 10 mM MgCl2, 1 mM thiamin pyrophosphate, 1 mM EDTA (all from (Sigma, St. Louis, Mo.), 25 mM sodium acetate (Fisher Scientific, Fair Lawn N.J.), and 0.25 ml of a BudB preparation. The mixture was stirred under a pH meter at room temperature. As the reaction proceeded, CO2 was evolved, and the pH rose, Pyruvic acid (Alfa, Ward Hill, Mass.) was added slowly via peristaltic pump to keep the pH between 6 and 7. As the pH rises, the enzyme reaction slows, but if it is allowed to fall below 6, decarboxylation of acetolactic acid becomes a problem. When the reaction was complete, the mixture was stored at −80° C.

Synthesis of DHMB

DHMB was synthesized chemically from (S)-acetolactate. Three ml of a crude acetolactate preparation at ~0.8 M at pH ~8 was treated with 1.2 equiv $NaBH_4$ (Aldrich Chemical Co, Milwaukee, Wis.). The reaction was allowed to sit at room temperature overnight before being divided in two and desalted in two portions on a 60 cm×1 cm diameter column of Biogel P-2 (Bio-Rad, Hercules, Calif.) using water as the mobile phase. The fractions containing mixed DHMBs were concentrated by rotary evaporation and adjusted to pH 2.2 with sulfuric acid.

The diastereomers of DHMB were separated using an HPLC system (consisting of an LKB 2249 pump and gradient controller (LKB, now a division of General Electric, Chalfont St Giles, UK) and a Hewlett-Packard (now Agilent, Santa Clara, Calif.) 1040A UV/vis detector) with a Waters Atlantis T3 (5 um, 4.6×150 mm) run at room temperature in 0.2% aqueous formic acid, pH 2.5, at a flow rate of 0.3 mL/min,

with UV detection at 215 nm. "Fast" DHMB was eluted at 8.1 min and "slow" DHMB was eluted at 13.7 min, DHIV was not present. The pooled fractions were taken nearly to dryness, and coevaporated with toluene to remove residual formic acid. The residue was then dissolved in water and made basic with triethylamine (Fisher, Fair Lawn, N.J.).

Concentration Determination and Absolute Structure of DHMB

The concentration of purified DHMB solutions was determined as follows. The concentration was estimated based on the mmol acetolactate used in the $NaBH_4$ reduction. To portions of the DHMBs, a known quantity of sodium benzoate (made by dissolving solid benzoic acid (ACS grade, Fisher Scientific, Fair Lawn, N.J.) in aqueous NaOH)) was added to give two-component mixtures in (approximately) equimolar amounts. A similar sample of DHIV was also prepared from the solid sodium salt obtained via custom synthesis (Albany Molecular Research, Albany N.Y.). The samples were coevaporated several Limes with D_2O (Aldrich, Milwaukee, Wis.) and redissolved in D_2O. Integrated proton NMR spectra were obtained and used to determine the mole ratio of DHIV or DHMB to benzoate. Comparison of the NMR spectra of the DHMBs with the literature spectra for the free acids in $CDCl_3$ (Kaneko et al., *Phytochemistiy* 39: 115-120 (1995)) showed that fast DHMB was the erythro isomer. Since enzymatically synthesized acetolactate has the (5) configuration at C-2, the fast DHMB has the 2S, 3S configuration. Slow DHMB has the threo 2S, 3R configuration.

Dilutions of the NMR samples were also analyzed by LC/MS using separately prepared benzoic acid solutions as standards. Benzoic acid, DHIV, and the two isomers of DHMB were separated and quantified by LC/MS on a Waters (Milford, Mass.) AcquityTQD system, using an Atlantis T3 (part #186003539) column, as described above. Benzoic acid was detected at m/z=121 (negative ESI), and emerged at 2.05 min. The concentration of benzoate in the mixtures was within experimental uncertainty of the expected value. The experiment also showed that either isomer of DHMB had ~80% of the sensitivity of DHIV in LC/MS (i.e., MS peak area observed/nmol injected) throughout the response range of the instrument. Thus, if a DHIV standard is used to quantify DHMB found in cell extracts or in enzymatic reactions, the apparent DHMB concentrations need to be multiplied by 1.25.

Measuring Inhibition of KARI by DHMB

Purified KARI encoded by genes either from *Lactococcus lactis* (SEQ ID NO: 864), a derivative of *Pseudomonas fluorescens* KARI known as JEA1 (SEQ ID NO: 799; U.S. Appl. Pub No. 2010/0197519, which is herein incorporated by reference in its entirety), or a variant of *Anaerostipes caccae* KARI known as K9D3 (SEQ ID NO:788), were tested for their sensitivity to DHMB inhibition in spectrophotometric assays in a Shimadzu (Kyoto, Japan) UV160U instrument with a TCC240A temperature control unit, set at 30° C. The buffer was 0.1 M K+ Hepes, pH 6.8, containing 10 mM $MgCl_2$ and 1 mM EDTA. NADPH was present at 0.2 mM, and racemic acetolactate was present at either 3 mM or 0.725 mM (S) isomer. The rate of NADPH oxidation in the presence and absence of either fast or slow DHMB was measured. V_{max} for each sample was calculated from the observed rate and the known acetolactate K_M using the Michaelis-Menten equation. A volumetric K was estimated for each measurement in the presence of DHMB using the Michaelis-Menten equation as modified for competitive inhibition vs. acetolactate (the K_M term in the Michaelis-Menten equation is multiplied by $(1+[I]/K_i)$, and the equation is solved for K_i. The results were converted to mM upon completion of the NMR experiment and are shown in Table 32.

TABLE 32

K_I Values for KARI Inhibition by DHMB Isomers

Strain	Fast DHMB	Slow DHMB
JEA1	0.23 mM	0.23 mM
K9D3	0.3 mM	0.2 mM
L. lactis	2.8 mM	2.3 mM

Example 30

Inhibition of DHAD by DHMB

Purified dihydroxyacid dehydratase (DHAD) from *Staphococcus mutans* was tested for inhibition of conversion of dihydroxyisovalerate (DHIV) to 2-ketoisovalerate (2-KIV) by DHMB by using a modification of a colorimetric assay as described by Szamosi et al., *Plant Phys.* 101: 999W-1004 (1993). The assay took place in a 2 mL Eppendorf tube placed in a heating block maintained at 30° C. The assay mixture had a final volume of 0.8 mL containing 100 mM Hepes-KOH buffer, pH 6.8, 10 mM $MgCl_2$, 0.5-10 mM DHIV, 0-40 mM DHMB, and 18 μg DHAD. The assay was initiated by adding a 10× concentrated stock of substrate. Samples were removed (0.35 mL) at times 0.1 and 30 minutes, and the reaction was stopped by mixing into 0.35 mL 0.1 NHC with 0.05% 2,4-dinitrophenylhydrazine (Aldrich) in a second Eppendorf tube. After incubating 30 minutes at room temperature, 0.35 mL of 4N NaOH was added to the mixture, mixed, and centrifuged at 15,000×G for 2 minutes in a centrifuge (Beckman-Coulter Microfuge 18). The absorbance of the solution at 540 nm was then measured in a 1 cm pathlength cuvette using a Cary 300 Bio UV-Vis spectrophometer (Varian). Based on a standard curve using authentic 2-KIV (Fluka), 10D absorbance at 540 nm is produced by 0.28 mM 2-KIV. The rate of 2-KIV formation was measured in the presence and absence of either fast or slow DHMB. Both forms of DHMB behaved liked competitive inhibitors of DHIV. Their inhibition constants (Ki) were calculated from the Michaelis-Menten equation for simple competitive inhibition: $v=S*V_{max}/(S+K_M*(1+I/K_i)$, where v is the measured rate of 2-KIV formation, S is the initial concentration of DHIV, V_{max} is the maximum rate calculated from the observed rate at 10 mM DHIV and no DHMB, K_M is a previously measured constant of 0.5 mM, and I is the concentration of DHMB. The fast and slow isomers of DHMB had calculated inhibition constants of 7 mM and 5 mM, respectively.

Example 31

Identification of YMR226c Homologs

Homologs of the YMR226C gene of *Saccharomyces cerevisiae* were sought by BLAST searches of the GenBank

non-redundant nucleotide database (blast.ncbi.nlm.nih.gov/Blast.cgi), the Fungal Genomes BLAST Search Tool at the *Saccharomyces* Genome Database (www.yeastgenome.org/cgi-bin/blast-fungal.pl), and the BLAST Tool of the Genolevves Project (genolevures.org/blast.html#). Unique sequences from 18 yeast species showing high sequence identity to YMR226C were identified, and the complete ORF for these genes was recovered from the accessioned record in the associated database. The polypeptide sequences encoded by these ORFs were determined by the Translation feature of Vector NTI (Invitrogen, Carlsbad Calif.). The polynucleotide and polypeptide sequences are shown below in Table 33. The yeast species, nucleotide database accession number, and DNA and protein sequences are given in the Table. The *S. kluyveri* sequence is in the Genolevures database under the accession number given; the others are in GenBank. The percent identities between the sequences are shown in Table 34.

The 18 ORFs were aligned using AlignX (Vector NT; the gene encoding a putative NADP+-dependent dehydrogenase from *Neurospora crassa* (XM_957621, identified in the GenBank BLAST search using the YMR226C nucleotide sequence) was used as an outgroup. The resulting phylogenetic tree is shown in FIG. **11**, and a sequence alignment is shown in FIG. **12**.

The sequence identity of these homologs to YMR226C ranges from a minimum of 55% (*Yarrowia lipolytica* and *Schizosaccharomyces pombe*) to a maximum of 90% (*S. paradoxus*). A BLAST search also revealed a cDNA from *S. pastoriarius* (accession number CJ997537) with 92% sequence identity over 484 base pairs, but since this species is a hybrid between *S. bayanus* (whose YMR226C homolog shows 82% identity to the *S. cerevisiae* sequence), and because only a partial ORF sequence was available, this sequence was not included in the comparison. When the YMR226C sequence from the canonical laboratory strain 52880 was compared with the sequences from 12 other strains of *S. cerevisiae*, only 4 single-nucleotide polymorphisms are found (sequence identity 99.5%), indicating that this is a highly-conserved gene in that species.

TABLE 33

YMR226C Yeast Homologs

Species	Accession # (Date of database accession)	Nucleic acid SEQ ID NO:	Amino acid SEQ ID NO:
Saccharomyces paradoxus	AABY01000127 (Mar. 7, 2011)	698	699
Saccharomyces bayanus	AACA01000631 (Mar. 7, 2011)	700	701
Saccharomyces castellii	AACF01000116 (Mar. 7, 2011)	702	703
Saccharomyces mikatae	AACH01000019 (Mar. 7, 2011)	704	705
Ashbya gossypii	AE016819 (Mar. 7, 2011)	706	707
Candida glabrata	CR380959 (Mar. 7, 2011)	708	709
Debaryomyces hansenii	CR382139 (Mar. 7, 2011)	710	711
Scheffersomyces stipitis (formerly *Pichia stipitis*	XM_001387479 (Mar. 7, 2011)	712	713
Meyerozyma guilliermondii (formerly *Pichia guilliermondii*)	XM_001482184 (Mar. 7, 2011)	714	715
Vanderwaltozyma polyspora (formerly *Kluyveromyces polysporus*)	XM_001645671 (Mar. 4, 2011)	716	717
Candida dubliniensis	XM_002419771 (Mar. 7, 2011)	718	719
Zygosaccharomyces rouxii	XM_002494574 (Mar. 7, 2011)	720	721
Lachancea thermotolerans (formerly *Kluyveromyces thermotolerans*)	XM_002553230 (Mar. 4, 2011)	722	723
Kluyveromyces lactis	XM_451902 (Mar. 4, 2011)	724	725
Saccharomyces kluyveri	SAKL0H04730 (Mar. 7, 2011)	726	727
Yarrowia lipolytica	XM_501554 (Mar. 8, 2011)	728	729
Schizosaccharomyces pombe	NM_001018495 (Mar. 8, 2011)	730	731

TABLE 34

YMR226C Homolog Percent Identity

Species	Sm	Sb	Sca	Ag	Dh	Ss	Mg	Cd	Cg	Vp	Sk	Kl	Lt	Zr	Sce	Sp	Yl	Nc
Spa	88	82	70	64	62	62	58	57	67	68	68	69	68	68	90	55	55	56
Sm		82	70	64	60	62	58	56	67	69	68	70	68	69	86	57	56	57
Sb			71	63	59	62	58	53	67	66	68	70	69	67	82	56	56	58
Sca				60	62	61	60	59	65	69	69	71	64	70	69	57	53	54
Ag					56	60	57	54	59	61	62	62	62	62	63	54	55	55
Dh						64	62	61	61	63	62	61	59	63	62	57	57	53
Ss							68	64	61	62	62	64	62	63	62	56	58	58
Mg								60	57	58	60	60	59	62	59	57	57	56
Cd									57	62	59	60	54	60	58	57	53	49
Cg										69	70	68	67	67	66	55	56	55
Vp											71	72	67	70	71	58	52	51
Sk												77	71	72	69	53	54	54
Kl													71	72	71	56	52	54
Lt														69	69	53	60	58
Zr															69	58	55	55
Sce																55	55	56

TABLE 34-continued

	YMR226C Homolog Percent Identity																		
Species	Sm	Sb	Sca	Ag	Dh	Ss	Mg	Cd	Cg	Vp	Sk	Kl	Lt	Zr	Sce	Sp	Yl	Nc
Spo																	58	60
Yl																		61
Nc																		

Table 34 Key:
Saccharomyces paradoxus ("Spa");
Saccharomyces mikatae ("Sm");
Saccharomyces bayanus ("Sb");
Saccharomyces castellii ("Sca");
Ashbya gossypii ("Ag");
Debaryomyces hansenii ("Dh");
Scheffersomyces stipitis ("Ss");
Meyerozyma guilliermondii ("Mg");
Candida dubliniensis ("Cd");
Candida glabrata ("Cg");
Vanderwaltozyma polyspora ("Vp");
Saccharomyces kluyveri ("Sk");
Kluyveromyces lactis ("Kl");
Lachancea thermotolerans ("Lt");
Zygosaccharomyces rouxii ("Zr");
Saccharomyces cerevisiae ("Sce");
Schizosaccharomyces pombe ("Spo");
Yarrowia lipolytica ("Yl");
Neurospora crassa ("Nc")

Example 32

Prophetic

Screening of Aldehyde Dehydrogenases from *S. cerevisiae* for Ability to Convert Isobutyraldehyde to Isobutyric Acid Using Enzymatic Assays

This example demonstrates a method to determine which endogenous aldehyde dehydrogenases from *S. cerevisiae* can enzymatically convert isobutyraldehyde to isobutyric acid.

S. cerevisiae strains containing individual disruptions in the known aldehyde dehydrogenase enzymes are obtained from ATCC: BY4741 Δald2::kanMX4 (ATCC #4000753); BY4741 Δald3::kanMX4 (ATCC #4000752); BY4741 Δald4::kanMX4 (ATCC #4001671); BY4741 Δald5::kanMX4 (ATCC #4000213); and BY4741 Δald6::kanMX4 (ATCC #4002767).

The deletion strains above are first grown overnight in tubes containing 5 ml YPD media at 30° C. The 5 ml overnight cultures are transferred into 100 ml of medium in a 500 ml flask and incubated at 30° C. shaking at 220 rpm. The cultures are harvested when they reach 1 to 2 O.D. at 600 nm. The samples are washed with 10 ml of 20 mill Tris (pH 7.5) and then are resuspended in 1 ml of the same Tris buffer. The samples are transferred into 2.0 ml tubes containing 0.1 mm silica (Lysing Matrix B, MP biomedicals). The cells are then broken in a bead-beater (BIO101). The supernatant is obtained by centrifugation in a microfuge at 13,000 rpm at 4° C. for 30 minutes. Typically, 0.06 to 0.1 mg of crude extract protein is used in a single assay. Protein in the crude extracts was determined by Bradford assay with Coomassie stain.

Aldehyde dehydrogenase activity is measured following a protocol given by Sigma-Aldrich and by Bostian and Betts (Bostian, K. A. and Betts, G. F. (1978) Biochemical Journal 173, 773-786). Crude extracts from the deletion strains above and commercially available aldehyde dehydrogenase are tested using this method.

An alternative assay is to add isobutyraldehyde at concentrations from 1 to 30 mM to approximately 0.1 mg of crude extract protein in a sealed glass GC vials which are incubated at 30° C. for 30 minutes. The extracts are then centrifuged through 0.22 μm spin filters (Corning, Cat #8169) at 3000 rpm for 3 minutes, and the filtrate is transferred to a GC vial for GC-MS analysis. Isobutyraldehyde and isobutyric acid are detected.

The GC method utilizes an Agilent 7890 GC equipped with a 5975 mass spectrometer for detection, and a DB-1701 column (30 m×0.25 mm ID, 0.25 μm film) from Agilent (Santa Clara, Calif.). The carrier gas is helium at a constant flow rate of 1.0 mL/min; injector split is 1:10 at 250° C.; oven temperature is 40° C. for 1 min, 40° C. to 120° C. at 10° O/min, and 120° C. to 240° C. at 30° C./min. MS detection is used in full scan mode for identification and quantitation of isobutyraldehyde and isobutyric acid. Calibrated standard curves are generated for the following compounds: isobutyraldehyde, isobutyric acid, and isobutanol.

Example 33

Construction of Expression Vectors for Isobutanol Pathway Gene Expression in *S. cerevisiae*

pLH475-JEA1 Construction

The pLH475-JEA1 plasmid (SEQ ID NO: 419) was constructed for expression of ALS and KARI in yeast, pLH475-JEA1 is a pHR81 vector (ATCC #87541) containing the following chimeric genes:1) the CUP1 promoter (SEQ ID NO: 789), acetolactate synthase coding region from *Bacillus subtilis* (AlsS; SEQ ID NO: 790; protein SEQ ID NO: 791) and CYC1 terminator 2 (SEQ ID NO: 792); 2) an ILV5 promoter (SEQ ID NO: 793), Pf5.IlvC-JEA1 coding region and ILV5 terminator (SEQ ID NO: 794); and 3) the FBA1 promoter (SEQ ID NO: 795), *S. cerevisiae* KARI coding region (ILV5; SEQ ID NO: 796; protein SEQ ID NO: 797) and CYC1 terminator (SEQ ID NO: 798).

The Pf5.IlvC-JEA1 coding region is a sequence encoding KARI derived from *Pseudomonas fluorescens* but containing mutations, that was described in U.S. Patent Application Publication Nos. 2009/0163376 and 2010/0197519, which are herein incorporated by reference in their entireties. The Pf5.1IlvC-JEA1 encoded KARI (nucleic acid and amino acid

SEQ ID NOs: 799 and 800) has the amino acid changes as compared to the natural *Pseudomonas fluorescens* KARI.

Expression Vector pLH468

The pLH468 plasmid (SEQ ID NO:139) was constructed for expression of DHAD, KivD, and HADH in yeast.

Coding regions for *L. lactis* ketoisovalerate decarboxylase (KivD) and Horse liver alcohol dehydrogenase (HADH) were synthesized by DNA2.0 based on codons that were optimized for expression in *Saccharomyces cerevisiae* (SEQ ID NOs: 801 and 802, respectively) and provided in plasmids pKivDy-DNA2.0 and pHadhy-DNA2.0. The encoded proteins are SEQ ID NOs: 803 and 804, respectively. Individual expression vectors for KivD and HADH were constructed. To assemble pLH467 (pRS426::P_{TDH3}-kivDy-TDH3t), vector pNY8; also named pRS426.GPD-aid-GPDt, described in US Patent App. Pub. US2008/0182308, Example 17, which is herein incorporated by reference) was digested with AscI and SfiI enzymes, thus excising the GPD promoter and the aid coding region. A TDH3 promoter fragment (SEQ ID NO: 805) from pNY8 was PCR amplified to add an AscI site at the 5' end, and an SpeI site at the 3' end, using 5° primer OT1068 and 3' primer OT1067 (SEQ ID NOs: 806 and 807). The AscI/SfiI digested pNY8 vector fragment was ligated with the TDH3 promoter PCR product digested with AscI and SpeI, and the SpeI-SfiI fragment containing the codon optimized kivD coding region isolated from the vector pKivD-DNA2.0. The triple ligation generated vector pLH467 (pRS426::PTDH3-kivDy-TDH3t). pLH467 (SEQ ID NO: 808) was verified by restriction mapping and sequencing. pLH435 (pRS425::PGPM1-Hadhy-ADH1t) was derived from vector pRS425::GPM-sadB (SEQ ID NO: 809) which is described in U.S. Appl. Pub. No. 2009/0305363, Example 3, which is herein incorporated by reference. pRS425::GPM-sadB is the pRS425 vector (ATCC #77106) with a chimeric gene containing the GPM1 promoter (SEQ ID NO: 810), coding region from a butanol dehydrogenase of *Achromobacter xylosoxidans* (sadB; DNA SEQ ID NO: 811; protein SEQ ID NO: 812), and ADH1 terminator (SEQ ID NO: 444). pRS425::GPMp-sadB contains BbvI and PacI sites at the 5' and 3' ends of the sadB coding region, respectively. A NheI site was added at the 5' end of the sadB coding region by site-directed mutagenesis using primers OT1074 and OT1075 (SEQ ID NO: 813 and 814) to generate vector pRS425-GPMp-sadB-NheI, which was verified by sequencing. pRS425::P_{GPM1}-sadB-NheI was digested with NheI and Pad to drop out the sadB coding region, and ligated with the NheI-PacI fragment containing the codon optimized HADH coding region from vector pHadhy-DNA2.0 to create pLH435 (SEQ ID NO: 815). To combine KivD and HADH expression cassettes in a single vector, yeast vector pRS411 (ATCC #87474) was digested with SacI and NotI, and ligated with the SacI-SalI fragment from pLH467 that contains the P_{TDH3}-kivDy-TDH3t cassette together with the SalI-NotI fragment from pLH435 that contains the P_{GPM1}-Hadhy-ADH1t cassette in a triple ligation reaction. This yielded the vector pRS411::P_{TDH3}-kivDy-P_{GPM1}-Hadhy (pLH441, SEQ ID NO: 816), which was verified by restriction mapping.

In order to generate a co-expression vector for all three genes in the lower isobutanol pathway: ilvD, kivDy and Hadhy, we used pRS423 FBA ilvD(Strep) (SEQ ID NO: 817), as the source of the IlvD gene. This shuttle vector contains an F1 origin of replication (nt 1423 to 1879) for maintenance in *E. coli* and a 2 micron origin (nt 8082 to 9426) for replication in yeast. The vector has an FBA1 promoter (nt 2111 to 3108; SEQ ID NO: 795) and FBA1 terminator (nt 4861 to 5860; SEQ ID NO: 818). In addition, it carries the His marker (nt 504 to 1163) for selection in yeast and ampicillin resistance marker (nt 7092 to 7949) for selection in *E. coli*. The ilvD coding region (nt 3116 to 4828; SEQ ID NO: 819; protein SEQ ID NO: 820) from *Streptococcus mutans* UA159 (ATCC #700610) is between the FBA1 promoter and FBA1 terminator forming a chimeric gene for expression. In addition there is a lumio tag fused to the ilvD coding region (nt 48294849). The first step was to linearize pRS423 FBA ilvD(Strep) (also called pRS423-FBA(SpeI)-IlvD(*Streptococcus mutans*)-Lumio) with SacI and SacII (with SacII site blunt ended using T4 DNA polymerase), to give a vector with total length of 9,482 bp. The second step was to isolate the kivDy-hADHy cassette from pLH441 with SacI and KpnI (with KpnI site blunt ended using T4 DNA polymerase), which gives a 6,063 bp fragment. This fragment was ligated with the 9,482 bp vector fragment from pRS423-FBA(SpeI)-IlvD(*Streptococcus mutans*)-Lumio. This generated vector pLH468 (pRS423:: P_{FBA1}-ilvD (Strep)Lumio-FBA1t-P_{TDH3}-kivDy-TDH3t-P_{GPM1}-hadhy-ADH1t), which was confirmed by restriction mapping and sequencing.

Example 34

Construction of *S. cerevisiae* Host Strain Containing Modifications in Pyruvate Decarboxylase and Hexokinase 2

This example describes insertion-inactivation of endogenous PDC1, PDC5, and PDC6 genes of *S. cerevisiae*. PDC1, PDC5, and PDC6 genes encode the three major isozymes of pyruvate decarboxylase. Hexokinase 2, which is responsible for phosphorylation of glucose and transcriptional repression, is also inactivated. The resulting PDC/HXK2 inactivation strain was used as a host for expression vectors pLH475-JEA1 and pLH468.

Construction of pdc6::P_{GPM1}-sadB Integration Cassette and PDC6 Deletion

A pdc6::P_{GPM1}-sadB-ADH1t-URA3r integration cassette was made by joining the GPM-sadB-ADHt segment (SEQ ID NO: 821) from pRS425::GPM-sadB (described in U.S. Patent App. Pub. No. 2009/0305363, incorporated by reference) to the URA3r gene from pUC19-URA3r. pUC19-URA3r (SEQ ID NO: 822) contains the URA3 marker from pRS426 (ATCC #77107) flanked by 75 bp homologous repeat sequences to allow homologous recombination in vivo and removal of the URA3 marker. The two DNA segments were joined by SOE PCR (as described by Horton et al. (1989) *Gene* 77:61-68) using as template pRS425::GPM-sadB and pUC19-URA3r plasmid DNAs, with Phusion DNA polymerase (New England Biolabs Inc., Beverly, Mass.; catalog no. F-540S) and primers 114117-11A through 114117-11D (SEQ ID NOs: 823, 824, 825, 826), and 114117-13A and 114117-13B (SEQ ID NOs: 827 and 828).

The outer primers for the SOE PCR (114117-13A and 114117-13B) contained 5' and 3' ~50 bp regions homologous to regions upstream and downstream of the PDC6 promoter and terminator, respectively. The completed cassette PCR fragment was transformed into BY4700 (ATCC #200866) and transformants were maintained on synthetic complete media lacking uracil and supplemented with 2% glucose at 30° C. using standard genetic techniques (*Methods in Yeast Genetics,* 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp. 201-202). Transformants were screened by PCR using primers 112590-34G and 112590-34H (SEQ ID NOs: 829 and 830), and 112590-34F and 112590-49E (SEQ ID NOs: 831 and 832) to verify integration at the PDC6 locus with deletion of the PDC6 coding region. The URA3r marker was recycled by plating on synthetic

complete media supplemented with 2% glucose and 5-FOA at 30° C. following standard protocols. Marker removal was confirmed by patching colonies from the 5-FOA plates onto SD-URA media to verify the absence of growth. The resulting identified strain has the genotype: BY4700 pdc6::P_{GPM1}-sadB-ADH1t.

Construction of pdc1::P_{PDC1}-ilvD Integration Cassette and PDC1 Deletion:

A pdc1::P_{PDC1}-ilvD-FBA1t-URA3r integration cassette (SEQ ID NO: 833) was made by joining the ilvD-FBA1t segment from pLH468 to the URA3r gene from pUC19-URA3r by SOE PCR (as described by Horton of al. (1989) *Gene* 77:61-68) using as template pLH468 and pUC19-URA3r plasmid DNAs, with Phusion DNA polymerase (New England Biolabs Inc., Beverly, Mass.; catalog no. F-540S) and primers 114117-27A through 114117-27D (SEQ ID NOs: 823, 824, 825, 826).

The outer primers for the SOE PCR (114117-27A and 114117-27D) contained 5' and 3' ~50 bp regions homologous to regions downstream of the PDC1 promoter and downstream of the P001 coding sequence. The completed cassette PCR fragment was transformed into BY4700 pdc6::P_{GPM1}-sadB-ADH1t and transformants were maintained on synthetic complete media lacking uracil and supplemented with 2% glucose at 30° C. using standard genetic techniques (*Methods in Yeast Genetics,* 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp. 201-202). Transformants were screened by PCR using primers 114117-36D and 135 (SEQ ID NOs 834 and 835), and primers 112590-49E and 112590-30F (SEQ ID NOs 832 and 836) to verify integration at the PDC1 locus with deletion of the PDC1 coding sequence. The URA3r marker was recycled by plating on synthetic complete media supplemented with 2% glucose and 5-FOA at 30° C. following standard protocols. Marker removal was confirmed by patching colonies from the 5-FOA plates onto SD-URA media to verify the absence of growth. The resulting identified strain "NYLA67" has the genotype: BY4700 pdc6:: P_{GPM1}-sadB-ADH1t pdc1:: P_{PDC1}-ilvD-FBA1t.

HIS3 Deletion

To delete the endogenous HIS3 coding region, a his3::URA3r2 cassette was PCR-amplified from URA3r2 template DNA (SEQ ID NO: 837). URA3r2 contains the URA3 marker from pRS426 (ATCC #77107) flanked by 500 bp homologous repeat sequences to allow homologous recombination in vivo and removal of the URA3 marker. PCR was done using Phusion DNA polymerase and primers 114117-45A and 114117-453 (SEQ ID NOs: 838 and 839) which generated a ~2.3 kb PCR product. The HIS3 portion of each primer was derived from the 5' region upstream of the HIS3 promoter and 3' region downstream of the coding region such that integration of the URA3r2 marker results in replacement of the HIS3 coding region. The PCR product was transformed into NYLA67 using standard genetic techniques (*Methods in Yeast Genetics,* 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp. 201-202) and transformants were selected on synthetic complete media lacking uracil and supplemented with 2% glucose at 30° C. Transformants were screened to verify correct integration by replica plating of transformants onto synthetic complete media lacking histidine and supplemented with 2% glucose at 30° C. The URA3r marker was recycled by plating on synthetic complete media supplemented with 2% glucose and 5-FOA at 30° C. following standard protocols. Marker removal was confirmed by patching colonies from the 5-FOA plates onto SD-URA media to verify the absence of growth. The resulting identified strain, called NYLA73, has the genotype: BY4700 pdc6:: P_{GPM1}-sadB-ADH1t pdc1:: P_{PDC1}-ilvD-FBA1t Δhis3.

Deletion of HXK2:

A hxk2::URA3r cassette was PCR-amplified from URA3r2 template (described above) using Phusion DNA polymerase and primers 384 and 385 (SEQ ID NOs: 840 and 841) which generated a ~2.3 kb PCR product. The HXK2 portion of each primer was derived from the 5' region upstream of the HXK2 promoter and 3' region downstream of the coding region such that integration of the URA3r2 marker results in replacement of the HXK2 coding region. The PCR product was transformed into NYLA73 using standard genetic techniques (*Methods in Yeast Genetics,* 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp. 201-202) and transformants were selected on synthetic complete media lacking uracil and supplemented with 2% glucose at 30° C. Transformants were screened by PCR to verify correct integration at the HXK2 locus with replacement of the HXK2 coding region using primers N869 and N871 (SEQ ID NOs: 842 and 843). The URA3r2 marker was recycled by plating on synthetic complete media supplemented with 2% glucose and 5-FOA at 30° C. following standard protocols. Marker removal was confirmed by patching colonies from the 5-FOA plates onto SD-URA media to verify the absence of growth, and by PCR to verify correct marker removal using primers N946 and N947 (SEQ ID NOs: 844 and 845). The resulting identified strain named NYLA83 has the genotype: BY4700 pdc6:: P_{GPM1}-sadB-ADH1t pdc1::P_{PDC1}-ilvD-FBA1t Δhis3 Δhxk2.

Construction of pdc5::kanMX Integration Cassette and PDC5 Deletion

A pdc5::kanMX4 cassette was PCR-amplified from strain YLR134W chromosomal DNA (ATCC No. 4034091) using Phusion DNA polymerase and primers PDC5::KanMXF and PDC5::KanMXR (SEQ ID NOs: 846 and 847) which generated a ~2.2 kb PCR product. The PDC5 portion of each primer was derived from the 5' region upstream of the PDC5 promoter and 3' region downstream of the coding region such that integration of the kanMX4 marker results in replacement of the PDC5 coding region. The PCR product was transformed into NYLA83, and transformants were selected and screened as described above. The identified correct transformants named NYLA84 have the genotype: BY4700 pdc6:: P_{GPM1}-sadB-ADH1t pdc1:: P_{PDC1}-ilvD-FBA1t Δhis3 Δhxk2 pdc5::kanMX4. Plasmid vectors pLH468 and pLH475-JEA1 were simultaneously transformed into strain NYLA84 (BY4700 pdc6::P_{GPM1}-sadB-ADH1t pdc1::P_{PDC1}-ilvD-FBA1t Δhis3 Δhxk2 pdc5::kanMX4) using standard genetic techniques (*Methods in Yeast Genetics,* 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) and the resulting strain was maintained on synthetic complete media lacking histidine and uracil, and supplemented with 1% ethanol at 30° C.

Example 35

Prophetic

Construction of *S. cerevisiae* Host Strain Containing Modifications in Pyruvate Decarboxylase Hexokinase 2, and Aldehyde Dehydrogenase

This example describes inactivation of one or more aldehyde dehydrogenases that abolish or reduce formation of isobutryic acid. Disruption of ALD2 is used as an example, but the method could be used for disruption of any aldehyde

dehydrogenase. The resulting NYLA84-derived strain is used as a host for expression vectors pLH475-JEA1 and pLH468.

Deletion of ALD2:

A ald2::URA3r cassette is PCR-amplified from URA3r2 template (described above) using Phusion DNA polymerase and primers T001 and T002 (SEQ ID NOs: 848 and 849) which generates a ~2.3 kb PCR product. The ALD2 portion of each primer is derived from the 5' sequence and 3' sequence of the ALD2 gene such that integration of the URA3r2 marker results in replacement of the ALD2 coding region. The PCR product is transformed into NYLA84 using standard genetic techniques (*Methods in Yeast Genetics,* 2005, Cold Spring Harbor Laboratory Press, Cod Spring Harbor, N.Y., pp. 201-202) and transformants are selected on synthetic complete media lacking uracil and supplemented with 1% ethanol at 30° C. Transformants are screened by PCR to verify correct integration at the ALD2 locus with replacement of the ALD2 coding region using primers T003 and T004 (SEQ ID NOs: 850 and 851). The URA3r2 marker is recycled by plating on synthetic complete media supplemented with 1% ethanol and 5-FOA at 30° C. following standard protocols. Marker removal is confirmed by patching colonies from the 5-FOA plates onto SE-URA media to verify the absence of growth, and by PCR to verify correct marker removal using primers T004 and T005 (SEQ ID NOs: 851 and 852). The resulting identified strain is named NYLA84 Δald2 and is of the genotype: BY4700 pdc6:: P_{GPM1}-sadB-ADH1t pdc1:: P_{PDC1}-ilvD-FBA1t Δhis3 Δhxk2 Δald2.

Example 36

Production of Isobutanol in Recombinant *S. cerevisiae* in NYLA84 HPLC Method

Analysis for glucose and fermentation by-product composition is well known to those skilled in the art. For example, one high performance liquid chromatography (HPLC) method utilizes a Shodex SH-1011 column with a Shodex SH-G guard column (both available from Waters Corporation, Milford, Mass.), with refractive index (RI) detection. Chromatographic separation is achieved using 0.01 M H_2SO_4 as the mobile phase with a flow rate of 0.5 mL/min and a column temperature of 50° C. Isobutanol retention time is 47.6 minutes.

Production of Isobutanol in Recombinant *S. cerevisiae* in NYLA84

The purpose of this example is to describe the production of isobutanol in the yeast strain NYLA84. The yeast strain comprises deletions of PDC1, PDC5, and PDC6, genes encoding three isozymes of pyruvate decarboxylase, and deletion of HXK2 encoding hexokinase 2. The strain also contains constructs for heterologous expression of AlsS (acetolactate synthase), KARI (keto acid reductoisomerase), DHAD (dihydroxy acid dehydratase), KivD (ketoisovalerate decarboxylase), SadB (secondary alcohol dehydrogenase), and HADH (horse liver alcohol dehydrogenase).

Strain Construction

Plasmids pLH468 and pLH475-JEA1 were introduced into NYLA84, by standard PEG/lithium acetate-mediated transformation methods. Transformants were selected on synthetic complete medium lacking glucose, histidine and uracil. Ethanol (1% v/v) was used as the carbon source. After three days, transformants were patched to synthetic complete medium lacking histidine and uracil supplemented with both 2% glucose and 0.5% ethanol as carbon sources. Freezer vials were made by dilution of log-phase cultures with 45% glycerol to a final glycerol concentration of 15% (w/v).

Production of Isobutanol

Freezer vials were used to inoculate 80 ml of synthetic complete medium lacking histidine and uracil supplemented with both 2% glucose and 0.5% ethanol as carbon sources.

Fermentation Conditions:

A 1 liter fermenter was prepared and sterilized with 0.4 L water. After cooling, filter sterilized medium was added to give the following final concentrations in 800 mL post-inoculation:

Medium (Final Concentration):

6.7 g/L, Yeast Nitrogen Base w/o amino acids (Difco)

2.8 g/L, Yeast Synthetic Drop-out Medium Supplement Without Histidine, Leucine, Tryptophan and Uracil (Sigma Y2001)

20 mL/L of 1% (w/v) L-Leucine

4 mL/L of 1% (w/v) L-Tryptophan

1 mL/L ergosterol/tween/ethanol solution

0.2 mL/L Sigma DF204

10 g/L glucose

The fermenter was set to control at pH 5.5 with KOH, 30% dO, temperature 30° C., and airflow of 0.2 SLPM (or, 0.25 vvm). At inoculation, the airflow was set to 0.01 SLPM initially, then increased to 0.2 SLPM once growth was established. Glucose was maintained at 5-15 g/L throughout by manual addition.

Air was continuously supplied to the fermentor at 0.25 vvm. Continuous aeration led to significant stripping of isobutanol from the aqueous phase of the fermentor. To quantify the loss of isobutanol due to stripping, the off-gas from the fermentor was directly sent to a mass spectrometer (Prima dB mass spectrometer, Thermo Electron Corp., Madison, Wis.) to quantify the amount of isobutanol in the gas stream. The isobutanol peaks at mass to charge ratios of 74 or 42 were monitored continuously to quantify the amount of isobutanol in the gas stream. Glucose and organic acids in the aqueous phase were monitored during the fermentation using HPLC. Glucose was also monitored quickly using a glucose analyzer (YSI, Inc., Yellow Springs, Ohio). Isobutanol in the aqueous phase was quantified by HPLC as described in HPLC Method herein above after the aqueous phase was removed periodically from the fermentor. The effective titer, corrected for the isobutanol lost due to stripping, was 7.5 g/L. The titer of isobutyric acid was 1.28 g/L (FIG. **14**).

Example 37

Prophetic

Production of Isobutanol in Recombinant *S. cerevisiae* in NYLA84 Δald2

The purpose of this example is to describe the production of isobutanol in the yeast strain NYLA84. Disruption of ALD2 is used as an example, but the example could be used for disruption of any aldehyde dehydrogenase. The NYLA84 Δald2 yeast strain comprises deletions of PDC1, PDC5, and PDC6, genes encoding three isozymes of pyruvate decarboxylase, deletion of HXK2 encoding hexokinase 2, and deletion of ALD2 encoding aldehyde dehydrogenase. The strain also contains constructs for heterologous expression of AlsS (acetolactate synthase), KARI (keto acid reductoisomerase), DHAD (dihydroxy acid dehydratase), KivD (ketoisovalerate decarboxylase), and SadB (secondary alcohol dehydrogenase).

Strain Construction

Plasmids pLH468 and pLH475-JEA1 are introduced into NYLA84 Δald2, by standard PEG/lithium acetate-mediated

transformation methods. Transformants are selected on synthetic complete medium lacking glucose, histidine and uracil. Ethanol (1% v/v) is used as the carbon source. After three days, transformants are patched to synthetic complete medium lacking histidine and uracil supplemented with both 2% glucose and 0.5% ethanol as carbon sources. Freezer vials are made by dilution of log-phase cultures with 45% glycerol to a final glycerol concentration of 15% (w/v).

Production of Isobutanol

Freezer vials are used to inoculate 80 ml of synthetic complete medium lacking histidine and uracil supplemented with both 0.25% glucose and 0.5% ethanol as carbon sources. A 1 liter fermenter is prepared and sterilized with 0.4 L water. After cooling, filter sterilized medium is added to give the following final concentrations in 800 mL post-inoculation:

Medium (Final Concentration):

6.7 g/L, Yeast Nitrogen Base w/o amino acids (Difco)

2.8 g/L, Yeast Synthetic Drop-out Medium Supplement Without Histidine, Leucine, Tryptophan and Uracil (Sigma Y2001)

20 mL/L of 1% (w/v) L-Leucine

4 mL/L of 1% (w/v) L-Tryptophan

1 mL/L ergosterol/tween/ethanol solution

0.2 mL/L Sigma DF204

10 g/L glucose

The fermenter is set to control at pH 5.5 with KOH, 30% dθ, temperature 30° C., and airflow of 0.2 SLPM (or, 0.25 vvm). At inoculation, the airflow is set to 0.01 SLPM initially, then increased to 0.2 SLPM once growth is established. Glucose is maintained at 5-15 g/L throughout by manual addition.

To quantify the loss of isobutanol due to stripping, the off-gas from the fermentor is directly sent to a mass spectrometer (Prima dB mass spectrometer, Thermo Electron Corp., Madison, Wis.) to quantify the amount of isobutanol in the gas stream. The isobutanol peaks at mass to charge ratios of 74 or 42 are monitored continuously to quantify the amount of isobutanol in the gas stream. Glucose and organic acids in the aqueous phase are monitored during the fermentation using HPLC. Glucose is also monitored quickly using a glucose analyzer (YSI, Inc., Yellow Springs, Ohio). Isobutanol and isobutyric acid in the aqueous phase are quantified by HPLC after the aqueous phase was removed periodically from the fermentor.

Example 38

Analysis of Isobutyric Acid Production

Strains were inoculated into synthetic complete medium, minus histidine and uracil, supplemented with 0.05% ethanol. Once cultures had reach stationary phase they were subcultured into fresh medium (starting OD=0.2). For PNY2205 (Example 13), medium was supplemented with 0.05% ethanol to satisfy the C2 requirement observed in PDC KO yeast that do not have the phosphoketolase pathway. For PNY2209 (Example 13), cells were subcultured into media with and without ethanol. For the ald6Δ clones (PNY2216 and isogenic clones, Example 24), medium without ethanol was used. Cultures were grown in screw capped shake flasks (20 ml medium in 125 ml flasks). Culture supernatants were collected immediately after inoculation and again after 48 hours. These culture supernatants were analyzed by HPLC (described in US20070092957, incorporated herein by reference) to determine glucose consumption and isobutyric acid concentration.

TABLE 35

Molar yield of isobutyric acid in ald6Δ strains.

Strain	Isobutyric acid molar yield (mole/mole glucose consumed)
PNY2205*	0.09
PNY2209*	0.07
PNY2209	0.09
PNY2216 and 5 other isogenic clones	0.03

*Indicates culture medium was supplemented with 0.05% ethanol

Example 39

Increased Production of Isobutanol and Decreased By-Product Production in New Strains

The purpose of this example is to describe small-scale culturing experiments with the newly constructed strains using plasmid pK9G9.OLE1p.ilvD to supply the remaining isobutanol pathway genes. New host strains PNY1528, PNY2243, PNY2237 and PNY2238 were transformed with plasmid pK9G9.OLE1p.ilvD and tested for isobutanol production. Transformants were obtained by selection on synthetic complete medium minus uracil with 1% ethanol as the carbon source. Transformants were patched on synthetic complete medium minus uracil with 2% glucose and 0.05% ethanol as carbon sources. Patches were used to inoculate liquid medium (synthetic complete minus uracil with 0.3% glucose and 0.3% ethanol as carbon sources). To test isobutanol production, liquid cultures were sub-cultured into synthetic complete medium minus uracil containing 2% glucose and 0.05% ethanol as carbon sources that also contained BME vitamin mix (Sigma Cat. No. B6891). Cultures were incubated in sealed serum vials (10 ml medium in 15 ml vials) at 30° C. with shaking (250 rpm in an Infors Multitron shaker). After 48 hours, culture medium was filtered (Spin-X column) and analyzed by HPLC (as described previously in US App. Pub. No. 20070092957). Molar yield of the pathway by-product isobutyrate was decreased by 50% in strains carrying the ald6Δ. Strains based on PNY2238 were found to have higher glucose consumption and isobutanol titer (done K results shown in the table below).

TABLE 36

Molar Yield of isobutyric acid in the ald6Δ strains

Strain	Relevant phenotype	Isobutyric acid molar yield (mole/mole glucose consumed)
PNY2205*	ALD6+, C2-dependent	0.09
PNY2209*	ALD6+, C2-independent	0.07
PNY2209	ALD6+, C2-independent	0.09
PNY2216 and 5 other isogenic clones	ALD6−, C2-independent	0.03

*indicates the culture medium was supplemented with 0.05% ethanol

All strains contained plasmid pK9G9.OLE1p.ilvD. For PNY2237- and PNY2238-derived strains, the data presented are an average of two biological replicates.

Example 40

Construction of a Site-Saturation Gene Library and Screening the Isobutanol Production of the Resultant Variants in PNY2259

The reverse primer mixture (called K9_309r in this example) containing primers encoding all 20 individual

amino acid changes at position 309 (Table 37) and the forward primer K9_131T080211f: GGACTTGATGTCACTATGATC (called K9_131Tf in this example) were employed to create a single-site saturation library targeting the position of 309 of K9 KARI. A plasmid containing variant K9SB2 (pHR81-ILV5p.K9SB2.TEF1(M4)p.ilvD (SEQ ID NO: 930, also called "pLH744").

In brief, a megaprimer was prepared through a regular PCR. The megaprimer PCR mixture consisted of 45 μl of SuperMix (Invitrogen, Carlsbad, Calif., #10572063), 2.0 μl K9_131Tf (20 μM), 2.0 μl K9_309r (20 μM) and 1.0 μl template (50 ng/μl). The PCR program for making the megaprimer is: the starting temperature was 95° C. for 1.0 min followed by 35 heating/cooling cycles. Each cycle consisted of 95° C. for 20 sec, 55° C. for 20 sec, and 72° C. for 1.0 min. The megaprimer was then used to introduce mutation into K9SB2 using the same procedure as shown in Example 5. Clones from the single-site saturation library were sequenced.

The resultant unique variants together with pLH744 were analyzed for isobutanol production and by-product formation in yeast (triple for each mutant). Yeast pathway strains were made in PNY2259 (MATa ura3Δ::loxP his3Δ pdc6Δ pdc1Δ::P[PDC1]-DHAD|ilvD_Sm-PDC1t-P[FBA1]-ALS|alsS_Bs-CYC1t pdc5Δ::P[PDC5]-ADH|sadB_Ax-PDC5t gpd2Δ::loxP fra2Δ::P[PDC1]-ADH|adh_Hl-ADH1 t adh1Δ::UAS(PGK1)P[FBA1]-kivD_Lg(y)-ADH1t yprcΔ15Δ::P[PDC5]-ADH|adh_Hl-ADH1t ymr226cΔ ald6Δ::loxP; Example 22) host by transforming the KARI vectors. The transformed cells were plated on synthetic medium without histidine or uracil (1% ethanol as carbon source). Three transformants were transferred to fresh plates of the same media. The transformants were tested for isobutanol production under anaerobic conditions in 48-well plates (Axygen, Union City, Calif. #391-05-061).

Yeast colonies from the transformation on SE-Ura plates appeared after 5-7 days. The three colonies from each variant were patched onto fresh SE-Ura plates, and incubated at 30° C. for 3 days.

Growth Media and Procedure

Two types of media were used during the growth procedure of yeast strains: an aerobic pre-culture media and an anaerobic culture media. AH chemicals were obtained from Sigma unless otherwise noted (St. Louis, Mo.)

Aerobic pre-culture media (SE-Ura): 6.7 g/L yeast nitrogen base without amino acids (Difco, 291940, Sparks, Md.), 1.4 g/L yeast synthetic drop-out medium supplement without histidine, leucine, tryptophan and uracil, 0.2% ethanol, 0.2% glucose, 0.01% w/v leucine, 0.002% w/v histidine, and 0.002% w/v tryptophan.

Anaerobic culture media (SEG-Ura-His): 50 mM MES (pH 5.5, 6.7 g/L yeast nitrogen base without amino acids (Difco, 291940, Sparks, Md.), 1.4 g/L yeast synthetic drop-out medium supplement without histidine, leucine, tryptophan and uracil, 0.1% ethanol, 3% glucose, 0.01% leucine, 0.002% w/v histidine, 0.002% tryptophan, 30 mg/L nicotinic acid, 30 mg/L thiamine and 10 mg/L ergosterol made up in 50150 v/v Tween/ethanol solution.

The patched cells were inoculated into 48-well plates. Each well contains 1.5 ml aerobic media. The plates were covered with permeable foils and grown at 30° C. with shaking overnight. The cell density (OD_{600}) was then measured. The amount of cells to make 1.5 ml of cell suspension (in anaerobic media) with the final OD_{600}=0.2 for each well were calculated and 1.5 ml cell suspension was prepared with anaerobic media and added into each well. Oxygen in 48-well plates was removed using an anaerobic chamber following the manufacturer's protocol (Coy Laboratory Products Inc. Grass Lake, Mich.). Cells were then grown at 30° C. with shaking for two days. The cell density (OD_{600}) was then measured. The best grown mutants underwent the same two-day growth in 48-well plates for isobutanol production measurement. After two days' anaerobic growth, the cell density (OD_{600}) was then measured. Cells were centrifuged at 4,000 g for 5 min and the supernatant was collected for the isobutanol measurement using LC/MS.

LC/MS Analysis of Yeast Strains with K9 KARI Mutants

Samples were taken for LCMS analysis at the end of the anaerobic growth period. LCMS analysis was performed using a Waters AcQuity UPLC separations unit and AcQuity TQD triple quad mass spectrometer (Waters, Milford, Mass.) with a Waters AcQuity UPLC HSS T3 separations column (Waters, Milford, Mass.). Compounds were separated using a reverse phase gradient of water (+0.1% formic acid) and acetonitrile (+0.1% formic acid) starting with 99% aqueous and ending with 99% organic, at a flow rate of 0.5 mL/min. Chromatograms were analyzed using Waters Masslynx 4.1 software (Waters, Milford, Mass.). Micro molar yields for isobutanol were calculated using Waters Quanlynx software (Waters, Milford, Mass.) using a calibration curve of triplicate analyses of standards.

TABLE 37

Reverse Primers	
Targeted posifion(s) of K9-KARI Primers	
309	K9_309I_111711r: CTTTCTCATAGCCTTAATGTGGAC (SEQ ID NO: 884)
	K9_309L_111711r: CTTTCTCATAGCCTTTAAGTGGAC (SEQ ID NO: 885)
	K9_309Y_111711r: CTTTCTCATAGCCTTATAGTGGAC (SEQ ID NO: 886)
	K9_309C_111711r: CTTTCTCATAGCCTTACAGTGGAC (SEQ ID NO: 887)
	K9_309W_111711r: CTTTCTCATAGCCTTCCAGTGGAC (SEQ ID NO: 888)
	K9_309P_111711r: CTTTCTCATAGCCTTTGGGTGGAC (SEQ ID NO: 889)
	K9_309H_111711r: CTTTCTCATAGCCTTATGGTGGAC (SEQ ID NO: 890)
	K9_309Q_111711r: CTTTCTCATAGCCTTTTGGTGGAC (SEQ ID NO: 891)

TABLE 37 -continued

Reverse Primers	
Targeted posifion(s) of K9-KARI Primers	
K9_309M_111711r:	CTTTCTCATAGCCTTCATGTGGAC (SEQ ID NO: 892)
K9_309N_111711r:	CTTTCTCATAGCCTTATTGTGGAC (SEQ ID NO: 893)
K9_309V_111711r:	CTTTCTCATAGCCTTAACGTGGAC (SEQ ID NO: 894)
K9_309A_111711r:	CTTTCTCATAGCCTTAGCGTGGAC (SEQ ID NO: 895)
K9_309D_111711r:	CTTTCTCATAGCCTTATCGTGGAC (SEQ ID NO: 896)
K9_309E_111711r:	CTTTCTCATAGCCTTTTCGTGGAC (SEQ ID NO: 897)
K9_309G_111711r:	CTTTCTCATAGCCTTACCGTGGAC (SEQ ID NO: 898)
K9_309S_111711r:	CTTTCTCATAGCCTTAGAGTGGAC (SEQ ID NO: 899)
K9_309T_111711r:	CTTTCTCATAGCCTTAGTGTGGAC (SEQ ID NO: 900)
K9_309R_111711r:	CTTTCTCATAGCCTTTCTGTGGAC (SEQ ID NO: 901)
K9_309K_111711r:	CTTTCTCATAGCCTTCTTGTGGAC (SEQ ID NO: 902)

TABLE 38

List of some variants with OD_{600} and isobutanol titer (mM) after two days anaerobic growth

Variant	Amino Acid Seq ID No:	Repeat	OD_{600}	Isobutanol titer (mM)
K9C4	927	1	0.5502	76.99
		2	0.6578	84.05
		3	0.7301	98.91
K9C8	928	1	0.6887	116.57
		2	0.5309	78.77
		3	0.6859	102.49
K9SB2	427	1	0.6314	88.39
		2	0.5977	81.18
		3	0.2325	44.60

Example 41

Construction of K9SB2 SH (K9SB2 Short), a Truncated Version of K9SB2

A gene encoding a version of K9SB2 lacking the first five N-terminal amino acids (MEECK) was prepared by PCR with the Phusion® High-Fidelity PCR Kit (Catalog #E0553L, New England Biolabs). Primers Kshort1 (AAACCGGTTTAAACAGTATGGCTAAGATTTACTACCAAGAAGACTG; SEQ ID NO: 903) and YGrev (TTCTGTTTTATCAGACCGCTTC; SEQ ID NO: 904) were synthesized by Integrated DNA Technologies, Inc (Coralville Iowa). Other than the primers, dNTP mix (Cat#18427-013, Invitrogen, Carlsbad, Calif.), template, and ddH_2O, reagents used here were supplied with the kit indicated above. The PCR mixture consisted of 1 μl of K9SB2 in a pBAD.KARI plasmid (20 ng/μl; plasmid preparation described in Example 17; SEQ ID NO: 428), 2 μl of each primer (20 μM stocks), 10 μl of 5× Phusion HF Buffer, 2 μl of 5 mM dNTP mix, 0.5 μl polymerase, and 28 μl of ddH_2O. The following conditions were used for the PCR reaction: The starting temperature was 98° C. for 2 min followed by 30 heating/cooling cycles. Each cycle consisted of 98° C. for 10 sec, 48° C. for 30 sec, and 72° C. for 1.5 min. At the completion of the temperature cycling, the sample was kept at 72° C. for 10 min more, and then held awaiting sample recovery at 4° C. The reaction product was separated from the template via agarose gel electrophloresis (1% agarose, 1×TBE buffer) and recovered using the Illustra GFX™ PCR DNA and Gel Band Purification Kit (Cat#28-9034-70, GE Healthcare Sciences, Piscataway, N.J.) as recommended by the manufacturer. The purified PCR product was digested with PmeI and SfiI and ligated into the corresponding sites of pBAD-ps-JEA1 (SEQ ID NO: 905) and the sequence of the resultant construct K9SB2 SH in pBAD.KARI (SEQ ID NO: 929) was confirmed.

Example 42

Generation of Yeast Expression Plasmids for Studies of K9SB2 and Other KARI Enzymes

Construction of pHR81-ILV5p-K9SB2-TEF(M4)-IlvD (pLH744; SEQ ID NO: 930):

The K9SB2 gene was excised from pHR81-ILV5p-K9SB2 by PmeI and SfiI digestion, and ligated with pHR81-ILV5p-K9D3-TEF(M4)-IlvD (pBP2090) at PmeI and SfiI sites. The ligated vector was named pHR81-ILV5p-K9SB2-TEF(M4)-IlvD (pLH744), and this vector was confirmed by sequencing.

Construction of K9SB2_SH_DHAD (SEQ ID NO: 931):

The digested K9SB2_SH PCR product (described in example 41) was ligated into PmeI and SfiI sites of pLH744 and confirmed by sequencing.

Construction of K9SB2 SH 81 (SEQ ID NO: 932)

The digested K9SB2_SH PCR product (described in example 41) was ligated into PmeI and SfiI sites of pHR81-PIlv5-KARI-K9.G9 and confirmed by sequencing.

Construction of Plasmids for Yeast Expression of K9SB2 Derivatives

The yeast expression plasmids for variants listed in Table 39 (described in examples 17 and 21) were generated by subcloning the corresponding KARI genes from pBAD.KARI plasmids into the PmeI and SfiI sites of plasmid pLH744 and plasmid pHR81-PIlv5-KARI-K9.G9. The constructs were confirmed by sequencing.

TABLE 39

K9SB2 derivatives subcloned into yeast expression plasmids

K9SB2 Derivative	Alternate name	Amino Acid Sequence ID	Nucleic Acid Sequence ID
K9SB2-K90M	K9_David	431	432
K9SB2-G55D	K9_Eliza	433	946
K9SB2-Q91L	K9_Frank	440	947
K9SB2-A303D	K9_Grace	445	948
K9SB2-M94-V307I	K9_Ingrid	455	949
K9SB2-F67I	K9_Jarvis	437	950
K9SB2-A56G-K90N	K9_Kelly	452	951
K9SB2-G55C	K9_Norman	481	952
K9SB2-P135S	K9_Ophelia	488	953
K9SB2-F53L	K9_Pat	441	954
K9SB2-Q94I	K9_Quentin	495	955
K9SB2-F67L	K9_Ralph	496	956
K9SB2-K8N-K90M-T141I	K9_Sophia	502	957
K9SB2-E13V-M94I-T141I	K9_Tiberius	509	958
K9SB2-A56V	K9_Ursala	511	959
K9SB2-I84L	K9_Victor	514	960
K9SB2-W59C	K9_Watson	520	961
K9SB2-T93A	K9_Xavier	641	642

Construction of Plasmids for Yeast Expression of Truncated Versions of K9SB2 Derivatives

N-terminally truncated versions of K9SB2 derivatives were prepared as described in example 41, with modifications. The primer Kshort1 was replaced with the 5'-phosphorylated primer KPSH1 (AAACAGTATG GCT AAG ATT TAC TAC CAA GAA GAC TG; SEQ ID NO: 906), which was synthesized by Integrated DNA Technologies, Inc (Coralville Iowa). K9SB2 in the PCR was replaced by pooled mixtures of the variants (pBAD.KARI plasmids) listed in Table 39. The purified PCR products were digested with SfiI and ligated Into the PmeI and SfiI sites of pHR81-PIlv5-KARI-K9.G9. DNA sequencing with TempliPhi™ (GE Healthcare) and primers pHR81-F (ACACCCAGTATTTTC-CCTTTCC) and pHR81-Rev (CTA GTG TAC AGA TGT ATG TCG G) (SEQ ID NOs: 924 and 925) was performed to identify each truncated derivative. The identified variants are indicated in Table 40. The coding sequences for the truncated versions were subsequently subcloned into the PmeI and SfiI sites of plasmid pLH744 and confirmed by sequencing.

TABLE 40

Truncated versions of K9SB2 derivatives

Variant	Amino Acid Sequence ID	Nucleic Acid Sequence ID
K9_David_SH	196	263
K9_Eliza_SH	266	907
K9_Frank_SH	267	908
K9_Grace_SH	389	909
K9_Ingrid_SH	405	910
K9_Jarvis_SH	781	911
K9_Kelly_SH	782	912
K9_Norman_SH	783	913
K9_Ophelia_SH	835	914
K9_Pat_SH	853	915
K9_Quentin_SH	854	916
K9_Ralph_SH	855	917
K9_Ursala_SH	856	918
K9_Watson_SH	857	858
K9_Xavier_SH	859	919

Construction of K9_Zeke_SH (K9SB2-K90M-T93A) and K9_Annabel_SH (K9SB2-K90M-T93I)

K9_Zeke_SH (SEQ ID NO: 860, protein SEQ ID NO: 861) and K9_Annabel_SH (SEQ ID NO: 862, protein SEQ ID NO: 863) were derived from K9_David_SH via site directed mutagenesis employing the employing the QuikChange® Lightning Site-Directed Mutagenesis Kit (Catalog #210518; Agilent Technologies, Stratagene Products Division, La Jolla, Calif.). Except for the primers, templates, and ddH_2O, all reagents used here were supplied with the kit indicated above. Primers were synthesized by Integrated DNA Technologies, Inc (Coralville Iowa).

For K9_.Zeke_SH, primers employed were oMT93A (GATCCCAGATGAAATGCAGGCTGCCATG-TACAAAAACGACATCG; SEQ ID NO: 920)) and oMT93Arev (CGATGTCGTTTTTGTACATGGCAGCCT-GCATTTCATCTGGGATC; SEQ ID NO: 921). The reaction mixture contained 1 μl K9_David_SH in the pHR81-PIlv5-KARI-K9.G9-derived vector (20 ng/μl), 1 μl of each primer (150 ng/ul), 5 μl of 10× reaction buffer, 1 μl of dNTP mix, 1.5 μl QuikSolution, 1 ul QuikChange Lightning enzyme, and 38.5 μl ddH_2O. For K9_Annabel_SH, the primers were replaced with oMT93I (GATCCCAGATGAAATGCAGGC-TATCATGTACAAAAACGACATCG; SEQ ID NO: 922) and oMT93Irev (CGATGTCGTTTTTGTACATGATAGC-CTGCATTTCATCTGGGATC; SEQ ID NO: 923).

The following conditions were used for both reactions: The starting temperature was 95° C. for 2 min followed by 18 heating/cooling cycles. Each cycle consisted of 95° C. for 20 sec, 60° C. for 10 sec, and 68° C. for 6 min. At the completion of the temperature cycling, the samples held awaiting sample recovery at 4° C. 2 μl of the Dpn I was added to each reaction and the mixtures were incubated for 1 hour at 37° C. 2 μl of each mutagenic reaction was transformed into One Shot® Stbl3™ Chemically Competent *E. coli* (Invitrogen, Catalog #C7373-03) according to the manufacturer's instructions. The transformants were spread on agar plates containing the LB medium and 100 μg/ml ampicillin (Cat#L1004, Teknova Inc. Hollister, Calif.) and incubated at 37° C. overnight. Multiple transformants were then selected for TempliPhi™ Sequencing™ (GE Healthcare), which employed primers pHR81-F (ACACCCAGTATTTTCCCTTTCC; SEQ ID NO: 924) and pHR81-Rev (CTA GTG TAC AGA TGT ATG TOG G; SEQ ID NO: 925). Variants with the confirmed sequences (K9_Zeke_SH and K9_Annabel_SH in pHR81-PIlv5-KARI-K9.G9 derived vectors) were subcloned into the PmeI and SfiI sites of pLH744 (SEQ ID NO: 930).

Example 43

Isobutanol Production of K9SB2 and Derivatives Grown Under Anaerobic Conditions in 48-Well Plates

The yeast expression plasmids for K9SB2, K9SB2_SH, and K9SB2-T93A, prepared with vector derived from pLH744 as described in Example 42, were employed to evaluate isobutanol production in yeast grown under anaerobic conditions in a 48-well plate. Isobutanol production strains were made in host PNY2259 (MATa ura3Δ::loxP his3Δ pdc6Δ pdc1Δ::P[PDC1]-DHAD|ilvD_Sm-PDC1t-P[FBA1]-ALS|alsS_Bs-CYC1t pdc5Δ::P[PDC5]-ADH|sadB_Ax-PDC5t gpd2Δ::loxP fra2Δ::P[PDC1]-ADH|adh_Hl-ADH1t adh1Δ::UAS(PGK1)P[FBA1]-kivD_Lg(y)-ADH1t yprcΔ15Δ::P[PDC5]-ADH|adh_Hl-ADH1t ymr226cΔ ald6Δ::loxP) by transforming the plasmids containing the coding sequences for the KARI variants and plating on synthetic medium without uracil (1% ethanol as carbon source). Yeast colonies from the transfor-

mation on SE-Ura plates appeared after 3-5 days of incubation at 30° C. At least three colonies from each variant were patched onto fresh SE-Um plates and incubated at 30° C.

Yeast Cultivation Conditions:

Aerobic cultivation medium: SE-Ura medium with 2 g/l ethanol.

Anaerobic cultivation medium: SEG-Ura with 30 μl glucose and 1 g/l ethanol, supplemented with 10 mg/l ergosterol, 50 mM MES buffer (pH 5.5), 30 mg/l thiamine, and 30 mg/l nicotinic acid.

48-well plates: Axygen catalog #P-5ML-48-C—S, 5 ml/well total volume, culture volume of 1.5 ml/well.

Plates were covered with a permeable adhesive film (VWR; catalog number 60941-086) for aerobic cultivation. Plates were shaken at 225 rpm at 30° C. For anaerobic cultivation, freshly inoculated plates covered with permeable film were purged of oxygen by equilibration in an anaerobic chamber for 2 hours. The plate covers were then exchanged for adhesive aluminum covers (VWR; catalog number 89049-034) and each plate was placed into an airtight plastic box (Mitsubishi Gas Chemical America, Inc; New York, N.Y.; Catalog 50-25) along with a fresh oxygen scavenger pack (Mitsubishi Gas Chemical America, Inc; New York, N.Y.; Catalog 10-01). The entire assembly (plate(s) and oxygen scavenger pack inside a sealed airtight plastic box) was removed from the anaerobic chamber and shaken at 225 rpm at 30° C.

Experimental Protocol

Single yeast colonies on SE-Ura agar plates were streaked onto fresh SE-Ura agar plates and incubated at 30° C. until dense patches of cells had grown. Liquid precultures in 48-well plates were inoculated with loops of these cells for initial aerobic cultivation. After shaking overnight, the OD_{600} of each culture well was measured by transferring 0.15 ml of each well into a flat-bottom 96-well plate and measuring the absorbance of each well at 600 nm with a Molecular Devices (Sunnyvale, Calif.) plate reader. A linear transformation based on an experimentally-determined calibration line was applied to these plate reader-measured optical densities to convert them into comparable absorbance values for a cuvette-based spectrophotometer.

A calculated portion of each aerobic preculture well was inoculated into the corresponding well of a fresh 48-well plate with 1.5 ml of the SEG-Ura medium, to achieve an initial OD_{600} (in cuvette spectrophotometer absorbance units) of 0.2. In the process of inoculating the fresh plate, the aerobic preculture plate was centrifuged, the supernatant was removed from each well, and the cells in each well were resuspended in fresh SEG-Ura medium. This anaerobic cultivation plate was shaken for 3 days. The isobutanol concentration in the culture supernatants was measured by HPLC (Table 41).

TABLE 41

Isobutanol titers reached in the first anaerobic passaging cycle

Variant	# wells	Mean Isobutanol Titer (mM)	Standard Deviation of Isobutanol Titer (mM)
K9SB2	16	35.2	11.9
K9SB2_SH	8	67.4	12.2
K9SB2-T93A	8	40.8	9.1

A follow-up anaerobic cultivation was initiated from the first anaerobic cultivation as follows: A calculated portion of each anaerobic culture well was inoculated into the corresponding well of a fresh 48-well plate with 1.5 ml of the SEG-Ura medium, to achieve an initial OD_{600} (in cuvette spectrophotometer units) of 0.2. In the process of inoculating the fresh plate, the growth plate was centrifuged, the supernatant was removed from each well, and the cells in each well were resuspended in fresh SEG-Ura medium, in order to minimize carryover of metabolites from one cultivation to the next. The follow-up (second-cycle) anaerobic cultivation plate was shaken for 2 days. The isobutanol concentration in the culture supernatants was measured by HPLC (Table 42).

TABLE 42

Isobutanol titers reached in the second anaerobic passaging cycle

Variant	# wells	Mean Isobutanol Titer (mM)	Standard Deviation of Isobutanol Titer (mM)
K9SB2	16	67.6	10.8
K9SB2_SH	8	85.7	9.2
K9SB2-T93A	8	76.3	16.8

Example 44

Kinetic characterization of K9G9, K9SB2, and K9SB2_SH

K9G9, K9SB2, K9SB2_SH were overexpressed via pBAD.KARI plasmids in *E. coli* strain Bw25113 (ΔilvC) and purified for detailed measurement of the kinetic parameters.

Expression, purification and cofactor kinetic analyses were performed as described in example 18, with the following modifications. Expression cultures were grown in 20 mL of LB with 100 μg/mL ampicillin and 0.2% (w/v) arabinose in a 125 mL vented cap flask. The expression media was inoculated with 1110 volume of 8 hour culture. Expression cultures were grown for 18 hours for K9 SB2 and 24 hours for K9SB2_SH.

TABLE 43

Kinetic Parameters Comparing Reactions with NADH and NADPH

Variant	SEQ ID NO:	V_{max} NADPH, U/mg	K_m NADPH, μM	V_{max}/K_m NADPH, L/min * mg	V_{max} NADH, U/mg	K_m NADH, μM	V_{max}/K_m NADH, L/min * mg
K9G9	644	2.2	24.1	0.091	1.9	78.2	0.024
K9SB2	427	1.7	44.8	0.038	1.8	11.6	0.155
K9SB2_SH	637	1.7	109.9	0.015	1.7	13.3	0.128

Example 45

Isobutanol Production of K9SB2 and Derivatives

Isobutanol production analysis in 48-well plates was performed, as described, in example 42, with the following modifications. Aerobic and anaerobic cultivation media are the same as those described in example 19, but with 0.01% w/v histidine added. OD_{600} values of aerobic precultures were measured using a Cary 300 spectrophotometer (Agent Technology, Wilmington, Del.). A Heraeus Multifug X1R with a M-20 rotor (Thermo Scientific, Waltham, Mass.) was used to pellet the aerobic pre-culture cells and the spent cultivation media was discarded. The plates with cell pellets were transferred into the Coy Anaerobic Bag (Grass Lake, Mich.) and 100 μL of anaerobic cultivation media was added to each pellet. The anerobic cultivation media was allowed to degas for at least 24 hours prior to the 48-well plate receiving 1.5 mL aliquots; this process was performed inside the anaerobic bag. The anaerobic culture plate was inoculated with the appropriate volume of cell resuspension and covered with an adhesive aluminum foil. Plates were placed into a MCG 2.5 L AnaeroPack system (MCG, Japan). The box was sealed and removed from the anaerobic bag and placed in a 30° C. incubator for 80 hours with shaking at 220 rpm. Three transformants were analyzed per variant, six transformations were analyzed for K9D3 and K92B2 (Table 44).

TABLE 44

Isobutanol Titers

Variant	SEQ ID NO:	Isobutanol Titer, mM
K9D3	645	76.4 ± 9.7
K9SB2	427	93.8 ± 3.3
K9__Frank	440	27.8 ± 0.5
K9__Grace	445	89.4 ± 15.5
K9__Ingrid	455	88.3 ± 5.4
K9__Jarvis	437	91.8 ± 5.8
K9__Kelly	452	40.9 ± 4.5
K9__Norman	481	66.2 ± 10.6
K9__Ophelia	488	28.3 ± 5.3
K9__Pat	441	77.1 ± 25.6
K9__Quentin	495	80.1 ± 10.8
K9__Ralph	496	82.1 ± 21.3
K9__Sophia	502	25.3 ± 13.4
K9__Tiberius	509	11.4 ± 10.4
K9__Ursala	511	57.3 ± 26.2
K9__Victor	514	93.0 ± 11.4

Isobutanol production analysis in serum vials was performed for select variants as described in Example 19, with the following modifications. KARI variants were expressed in yeast from plasmids derived from pLH744, prepared as described in Example 42. Histidine was added to both the aerobic pre-culture and anaerobic growth media to a final concentration of 0.01% w/v. Three or four transformants were analyzed per variant (Table 45, 46, and 47).

TABLE 45

Isobutanol Titers Experiment 1

Variant	SEQ ID NO:	Passage	Hours	Isobutanol Titer, mM	Isobutanol/ Glycerol
K9SB2	427	1	75	28.2 ± 6.85	2.11 ± 0.16
K9SB2_SH	637	1	75	87.9 ± 1.04	2.49 ± 0.04
K9SB2	427	2	48	85.0 ± 4.35	2.67 ± 0.06
K9SB2_SH	637	2	48	97.4 ± 4.79	2.49 ± 0.07

TABLE 46

Isobutanol Titers Experiment 2

Variant	SEQ ID NO:	Passage	Hours	Isobutanol Titer, mM	Isobutanol/ Glycerol
K9SB2	427	1	44	30.2 ± 4.1	2.30 ± 0.15
K9SB2__SH	637	1	44	40.3 ± 5.6	2.41 ± 0.07
K9__David	431	1	44	36.2 ± 3.5	2.40 ± 0.10
K9__David__SH	196	1	44	41.9 ± 6.4	2.37 ± 0.04
K9__Grace	445	1	44	40.2 ± 5.5	2.30 ± 0.05
K9__Pat	441	1	44	29.0 ± 3.1	2.35 ± 0.10
K9SB2	427	2	46	59.2 ± 0.6	2.61 ± 0.03
K9SB2__SH	637	2	46	74.8 ± 3.0	2.58 ± 0.03
K9__David	431	2	46	63.8 ± 0.6	2.65 ± 0.06
K9__David__SH	196	2	46	76.7 ± 1.5	2.53 ± 0.04
K9__Grace	445	2	46	62.3 ± 3.1	2.52 ± 0.07
K9__Pat	441	2	46	50.1 ± 2.1	2.48 ± 0.07

TABLE 47

Isobutanol Titers

Variant	SEQ ID NO:	Passage	Hours	Isobutanol Titer, mM
K9SB2	427	2	50	66.2 ± 1.8
Annabel__SH	862	2	50	74.4 ± 2.4
Zeke__SH	860	2	50	76.8 ± 8.3

TABLE Z

HMM	A m->m	C m->i	D m->d	E i->m	F i->i	G d->m	H d->d	I b->m	K m->e	L	M	N	P	Q	R	S	T	V	W	Y	Position in alignment
	−650	*	−1463																		
1(Q)	−648	−1356	−136	−44	−1453	−1166	−219	−1455	321	−1417	−911	−227	−1496	3263	122	−643	−684	−1239	−1542	−1030	7100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−38	−5840	−6882	−894	−1115	−701	−1378	−650	*												
2(M)	−4231	−3929	−5216	−5402	−3438	−4370	−4528	−3232	−5113	−2613	5320	−5052	−4790	−4977	−4823	−4692	−4459	−3629	−4103	−4017	7200%
—	−147	−501	232	42	−382	397	104	−625	209	−467	−722	276	396	44	95	361	121	−368	−296	−251	
—	−3303	−3318	−325	−3473	−136	−701	−1378	*	*												
3(F)	−1308	−1104	−2227	−2120	3516	−2093	−244	−196	−1891	64	66	−1626	−2278	−1503	−1798	−1617	−1350	−389	305	1335	8600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−38	−5840	−6882	−894	−1115	−943	−1060	*	*												
4(A)	1616	−1744	1125	33	−2015	−1540	−262	−1686	937	−1765	−911	−252	−1658	154	−383	−488	640	−3	−2038	−1421	8700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−901	−7402	−1125	−894	−1115	−2352	−314	*	*												
5(C)	−346	2578	1084	−712	2092	−1540	−384	−167	−624	−482	125	−731	−1705	−451	−883	−631	−338	−50	−774	−133	8800%
—	−149	−500	235	43	−381	398	106	−626	210	−466	−721	275	394	45	96	359	118	−369	−295	−249	
—	−1009	−1006	−7567	−131	−3527	−1916	−444	*	*												
6(S)	800	−586	−1937	−1415	−821	−1740	−954	1279	−1204	−584	19	−1258	−1964	−1013	−1358	1715	−476	1117	−1320	−938	9000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−17	−6953	−7995	−894	−1115	−146	−3378	*	*												
7(K)	−956	−2411	−803	501	−2743	−1919	−558	−2483	2435	−2420	−1502	57	−2010	1146	458	829	224	−2040	−2577	−1913	9100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
8(V)	−2472	−2010	−5089	−4702	−2534	−4789	−4391	2241	−4574	−151	−1318	−4442	−4600	−4417	−4628	−4080	−82	3023	−3952	−3510	9200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
9(Y)	−4673	−3685	−5210	−5505	2423	−5069	−1332	−3424	−5065	−392	−2838	−3726	−4920	−3835	−4458	−4313	−4533	−3643	−581	4349	9300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
10(Y)	−2170	−2625	−2489	−2097	−1555	−2986	−1481	−2628	906	−2674	−2098	−2051	−3206	−1513	−1078	−2258	1039	−2435	−2009	4185	9400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
11(D)	−2498	−4412	3500	1042	−4581	−2437	−1765	−4500	733	−4361	−3682	515	−2961	−1429	−2799	−2158	−2558	−3974	−4550	−3541	9500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
12(K)	11	−2371	348	819	−2692	−535	−527	−2443	2294	−2387	−1461	590	−1960	−68	904	−67	−837	−1993	−2554	−1871	9600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
13(D)	−2663	−4633	3700	580	−4789	−2487	−1872	−4738	731	−4578	−3963	−1073	−3046	−1551	−2987	−2292	−2742	−4201	−4759	−3709	9700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
14(C)	2503	3193	−4266	−3818	−2010	−3276	−2896	762	−3517	−1437	−1051	−3233	−3509	−3212	−3411	−2499	−1792	1507	−2796	−2431	9800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
15(D)	−1363	−2905	2748	542	−3202	−2072	−920	−2977	290	−2912	−2023	1270	−2294	−489	−1186	53	1116	−2518	−3086	−2349	9900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

16(L)	−1268	−1113	−3338	−540	−1057	−2827	−1716	569	−2409	2299	−236	−2381	−2862	−2089	−2316	−232	−1213	1306	−1645	−1304	10000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
17(S)	−1350	−2877	588	1045	−3189	−496	−920	−2963	−628	−2901	−2011	1860	−2289	−489	−1184	2139	190	−2503	−3077	−2343	10100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−2336	−8139	−325	−894	−1115	−701	−1378	*	*												
18(G)	−454	−832	−968	−1110	−2112	3143	−1211	−2091	−1317	−2264	−1691	−978	−1499	−1202	−1421	−646	−774	−1550	−1916	−1919	10200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−38	−5840	−6882	−894	−1115	−3098	−179	*	*												
19(H)	−898	−1313	−545	−482	−320	−1336	4297	−1552	−160	−1493	−1035	−579	−1675	−363	−322	−934	−951	−1354	−725	107	10300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−38	−5840	−6882	−894	−1115	−3098	−179	*	*												
20(D)	−872	−1812	3234	432	−2215	−967	−433	−2172	−569	−2269	−1704	99	−1453	−184	−1141	−728	−973	−1814	−2146	−1646	10400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−38	−5840	−6882	−894	−1115	−3098	−179	*	*												
21(E)	−766	−1695	521	2831	−2050	−1029	−293	−1804	−118	−1919	−1331	69	−1441	−4	−527	−653	−814	−1512	−1988	−1505	10500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−38	−5840	−6882	−894	−1115	−3098	−179	*	*												
22(Y)	−1337	−1229	−1681	−1596	1268	−1957	121	−918	−1294	−769	−585	−1229	−2163	−1111	−1301	−1443	−1359	−932	592	3932	10600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−38	−5840	−6882	−894	−1115	−109	−3775	*	*												
23(I)	−2294	−1931	−4749	−4227	−1724	−4227	−3320	2306	−3952	1990	−634	−3878	94	−3538	−3812	−3411	−2247	1576	−2891	−2629	10700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
24(I)	−2801	−2299	−5406	−5003	−2108	−5164	−4649	3051	−4886	1593	−869	−4829	−4788	−4454	−4829	−4493	−2764	1435	−3781	−3585	10800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
25(K)	−234	−2632	306	−500	−3007	−2141	−719	−2712	2540	−2619	−1730	−778	−2231	2257	968	−1109	−1152	−2288	−2738	−2136	10900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
26(G)	−2184	−3900	796	392	−4174	2903	−1580	−4030	−1636	−3937	−3173	−967	−2810	−1	−2362	1069	−2220	−3530	−4130	−3229	11000%
—	−149	−501	233	42	−375	399	104	−625	210	−463	−722	276	396	44	96	358	116	−371	−296	−251	
—	−155	−3318	−9181	−3674	−118	−701	−1378	*	*												
27(K)	−3243	−3775	−4129	−2558	−4750	−3647	−1490	−4021	3681	−3617	−2982	−2368	−3580	−1076	1318	−3119	−2876	−3817	−3395	−3374	12600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
28(K)	−1684	−2925	−1665	−979	−3407	−2535	−923	−3021	2737	−2865	−2032	202	−2582	1301	804	−1564	1681	−2645	−2905	−2448	12700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
29(V)	−2623	−2122	−5300	−4990	−2769	−5101	−5131	2388	−4945	−1532	−1474	−4790	−4868	−4890	−5101	−4482	−2619	3219	−4505	−3990	12800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
30(A)	3309	−1828	−4057	−4294	−4382	656	−3657	−4147	−4169	−4428	−3497	−2821	−2904	−3694	−3937	−1470	59	−2957	−4610	−4522	12900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
31(V)	−2625	−2122	−5304	−4993	−2772	−5111	−5142	2881	−4950	−1532	−1474	−4796	−4873	−4896	−5108	−4492	−2621	2896	−4512	−3997	13000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
32(I)	−2790	−2287	−5403	−5009	−2155	−5170	−4698	3324	−4899	1175	−912	−4835	−4802	−4495	−4860	−4506	−2757	1192	−3838	−3622	13100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

33(G)	−4435	−4203	−5092	−5462	−5893	3834	−5028	−6627	−5765	−6297	−5970	−5141	−4804	−5546	−5385	−4727	−4815	−5862	−4924	−5849	13200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
34(Y)	−4838	−3766	−5229	−5579	1502	−5108	−1300	−3726	−5134	−3040	−3131	−3723	−4963	−3861	−4500	−4356	−4689	−3881	2986	4507	13300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
35(G)	−4435	−4203	−5092	−5462	−5893	3834	−5028	−6627	−5765	−6297	−5970	−5141	−4804	−5546	−5385	−4727	−4815	−5862	−4924	−5849	13400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
36(S)	−1473	−2007	−3647	−3780	−3430	−2363	−3314	228	−3616	−3373	−2876	−2840	−3093	−3395	−3541	3475	−1885	−2307	−3927	−3474	13500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
37(Q)	−4589	−4392	−3927	−4146	−5099	−4221	−4099	−5973	−3840	−5564	−5304	−4230	−4693	4575	−3826	−4704	−4772	−5612	−4577	−4751	13600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
38(G)	677	−2128	−3838	−4171	−4647	3536	−3816	−4506	−4340	−4749	−3857	−3009	−3149	−3871	−4137	−1784	−2005	−3297	−4725	−4735	13700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
39(H)	−2667	−3375	−2682	−2114	−3744	−3201	4738	−3782	−445	−3553	−2886	−2112	866	−1265	1506	−2614	−2557	−3469	−3282	−2908	13800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
40(A)	3631	−2768	−4492	−4815	−4888	−2992	−4271	−4781	−4818	−5025	−4365	−3727	−3728	−4477	−4545	−2567	−2762	−3852	−4724	−4942	13900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
41(H)	−3103	−3404	−2950	−2573	−783	−3679	4549	−3407	−1372	−3071	−2715	−2454	−3764	2546	−1428	−2990	−2976	−3308	2269	−295	14000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
42(A)	3357	−1795	−4134	−4277	−4057	−2118	−3548	−3549	−4035	−4024	−3192	−2817	−2900	−3608	−3823	217	−1660	−276	−4363	−4211	14100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
43(Q)	−1061	1950	−2044	−1475	−1236	−2372	−1154	−789	−1218	1062	1123	743	−2446	2895	−1441	−1392	−1005	−693	−1678	−1278	14200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
44(N)	−4000	−4117	−3389	−3749	−5073	−3911	−4123	−6022	−4503	−5797	−5419	4397	−4479	−4255	−4592	−4115	−4312	−5371	−4650	−4731	14300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
45(L)	−4414	−3800	−5638	−5628	−2290	−4980	−4628	−1886	−5423	3316	−1236	−5514	−4997	−4750	−5002	−5379	−4399	−2629	−3665	−3690	14400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
46(R)	−1731	−3015	275	−931	−3487	−2518	−973	−3116	2321	−2955	−2123	224	−2603	256	2808	−1596	−1613	−2730	−2995	−2515	14500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
47(D)	−2896	−4843	3855	944	−5037	−2600	−2082	−5082	−2528	−4903	−4373	−1209	−3196	−1786	−3536	−2501	−3007	−4517	−5004	−3956	14600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
48(S)	−1536	−2212	−2363	−2679	−4293	−2279	−3082	−4365	−3331	−4524	−3676	288	−3026	−2967	−3497	3508	−1962	−3259	−4477	−4066	14700%
—	−148	−500	232	44	−381	398	105	−627	211	−465	−721	275	393	45	95	360	118	−370	−295	−250	
—	−155	−3318	−9181	−2405	−302	−701	−1378	*	*												
49(G)	−2521	−3968	1232	−911	−4849	3373	−2126	−4854	−2535	−4752	−4136	−53	−3115	−1836	−3440	−2284	−2716	−4157	−4880	−3914	15400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

50(V)	−2767	−2324	−5232	−4770	396	−4827	−3784	−36	−4546	848	−611	−4472	−4518	−3980	−4367	−4081	−2716	3323	−3037	−2660	15500%
—	−148	−500	233	43	−381	399	106	−626	211	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−148	−3381	−9181	−203	−2928	−701	−1378	*	*												
51(D)	−1684	−3285	2735	2014	−3554	−2196	−1177	−3350	92	−3279	−2427	692	−2505	−770	−1595	−1483	−1666	332	−3460	−2676	15700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
52(V)	−3122	−2888	−5092	−5160	−3522	−4180	−4687	−905	−5060	−2626	−2570	−4662	−4579	−4940	−4923	−4013	−3297	3796	−4414	−4190	15800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
53(V)	369	366	−3075	−2462	−883	−2557	−1420	1415	378	−757	−117	−2098	−2610	−1809	−2037	−1630	1166	2145	−1385	−343	15900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
54(V)	−2624	−2122	−5302	−4991	−2772	−5108	−5139	2623	−4948	−1533	−1475	−4794	−4871	−4894	−5106	−4488	−2620	3088	−4511	−3996	16000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
55(G)	929	−2107	−3852	−4182	−4633	3492	−3809	−4486	−4335	−4732	−3835	−2997	−3132	−3863	−4127	−1761	−1982	−3275	−4720	−4725	16100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
56(L)	−3427	−2938	−5791	−5325	−1449	−5374	−4410	−543	−5063	3041	−255	−5207	−4820	−4126	−4691	−4757	−3351	883	−3184	−3234	16200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
57(R)	−3040	−3724	−3266	82	−4620	−3470	−1396	−3905	804	−3529	−2874	−2133	−3439	−978	3800	−2894	−2709	−3682	−3353	−3267	16300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
58(K)	31	−2412	−803	1532	−2743	−1920	−559	−2483	1772	−2421	−1503	−556	1229	727	1079	−566	−893	−2041	−2579	−1915	16400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
59(G)	−2671	−4661	1614	587	−4832	3103	−1901	−4803	−2269	−4648	−4047	421	−3049	−1587	−3230	−2297	−2766	−4245	−4850	−3752	16500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
60(S)	−1499	−2308	−1932	−1859	−4006	1604	−2121	−3754	1362	−3793	−2945	−1833	−2827	−1794	−1902	2738	−1771	−2970	−3910	−3479	16600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−247	−8139	−2699	−894	−1115	−701	−1378	*	*												
61(K)	1362	−2232	−619	−98	−2567	−427	−435	−2309	1599	−2265	−1349	1101	−1861	886	−512	833	−740	−1868	−2441	−1767	16700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−9	−7900	−8943	−894	−1115	−344	−2238	*	*												
62(S)	−1288	−1904	−3742	−4011	−4384	−2155	−3593	−4209	−3996	−4479	−3573	−2789	−2948	−3606	−3832	3517	228	−3028	−4600	−4451	16800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
63(W)	726	−873	−3261	−2634	1926	−2567	−1425	660	−2252	−701	−68	−2174	−2617	−1898	18	−1648	−972	983	4091	−958	16900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
64(E)	1527	−2404	212	1636	−2722	−1878	−556	−2474	1241	−2419	−1497	350	−1985	−100	−659	96	70	−2025	−2589	−1903	17000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
65(K)	−8	−2242	−895	770	−2502	−1963	−609	−2192	2589	22	−1353	−631	−2052	692	−617	−889	−906	−361	−2455	−1836	17100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
66(A)	3631	−2768	−4492	−4815	−4888	−2992	−4271	−4781	−4818	−5025	−4365	−3727	−3728	−4477	−4545	−2567	−2762	−3852	−4724	−4942	17200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

67(Q)	−1006	−2441	−869	1767	−2780	−1965	−586	−2510	1702	−2445	−1534	−603	−2052	1923	873	−888	236	−630	−2596	−1949	17300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
68(A)	1489	−2393	167	1234	−2711	−1873	−547	−2462	895	−2408	−1485	1161	−1977	−90	−648	666	141	−2014	−2577	−1892	17400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
69(D)	2104	−2898	2124	985	−3163	−2096	1397	−2935	−693	−2897	−2025	−723	−2329	−543	−1250	−1245	−1368	−2501	−3087	−530	17500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
70(G)	−2294	−2898	−2521	−2885	−4852	3641	−3456	−5042	−3796	−5094	−4356	365	−3545	−3376	−4005	−2451	−2700	−3996	−4706	−4575	17600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
71(F)	−2596	−2266	−4685	−4188	3199	−4136	1018	505	−3812	1986	−405	−3595	−3961	−3157	−3524	−3277	−2509	−1337	−1621	−840	17700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
72(K)	47	−2348	338	950	−2668	−1854	−512	−507	1721	−2364	−1438	−490	−1947	672	436	806	687	−1970	−2533	−1851	17800%
—	−149	−500	232	46	−381	399	105	−627	210	−466	−721	277	393	45	95	359	119	−370	−295	−250	
—	−155	−3318	−9181	−2159	−366	−701	−1378	*	*												
73(V)	−1810	−1639	−4149	−3689	−1869	−3417	−2822	29	−3369	320	−897	−3230	112	−3099	−3291	−2619	−767	3269	−2708	−2354	18400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
74(K)	847	−1093	−2131	−1554	304	127	−1127	−637	1445	645	1186	−1534	−2401	−1174	−1547	−764	−172	−528	−1519	1413	18500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
75(T)	−1284	−2794	1526	1290	−3096	−2041	1289	−2863	−548	−2808	−1914	−668	−2242	−427	−1095	1451	1827	−2411	−2986	−2264	18600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
76(V)	−1089	−957	−3143	−2535	−943	−2618	−1496	1052	−2198	−792	1859	−146	686	−1884	−2111	−1695	945	2346	−1458	−1106	18700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
77(W)	1606	−2321	−752	612	−2628	−323	−527	−2366	1480	−2331	−1416	−510	−789	−73	421	23	−829	−1936	2212	−1843	18800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
78(E)	−1509	−3540	1372	3127	−3861	−120	−1391	−3685	−1319	−3605	−2787	−900	−2659	−1005	−1976	−400	−655	−3194	−3790	−2957	18900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
79(A)	3390	−1868	−4092	−4341	−4332	−2153	−3680	−3942	−4157	−4333	−3471	−2869	−2948	−3730	−3919	−1525	931	−2894	−4580	−4483	19000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
80(V)	2003	−1721	−4449	−3995	−2160	−3763	−3240	1342	−3745	−1435	−1124	−3561	−3855	−3494	−3700	−2979	−58	2574	−3091	−2698	19100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
81(K)	1714	−2501	−959	446	−2858	−2043	−654	−2574	1964	−2506	−1609	−689	−2135	−203	1088	428	−1032	−2148	−2652	−2027	19200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
82(W)	265	−2347	815	432	−2663	634	−519	−2410	619	−2361	−1438	−495	−1952	1955	−609	−382	147	−1966	2858	−1853	19300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
83(A)	3391	−1860	−3998	−4279	−4411	−2128	−3684	−4207	−4197	−4490	−3565	−2837	−2929	−3729	−3959	706	−1718	−3001	−4636	−4534	19400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

84(D)	−2747	−4795	3813	396	−4912	−2496	−1935	−4905	−2324	−4735	−4166	−1079	−3082	603	−3296	−2353	−2844	−4347	−4929	−3809	19500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
85(V)	−2717	−2220	−5338	−4951	−2254	−5099	−4670	1963	−4844	1553	−1011	−4759	−4771	−4509	−4836	−4427	−2688	2741	−3899	−3628	19600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
86(V)	−2635	−2129	−5306	−4970	−2652	−5125	−5011	2554	−4915	−354	−1368	−4781	−4852	−4798	−5038	−4487	−2622	3019	−4355	−3902	19700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
87(M)	−1340	−1208	−3317	−2708	−968	−2860	−1708	577	−2346	932	4131	−2382	−2878	250	−2265	−228	−1278	−506	−1629	−1313	19800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
88(I)	−2566	−2177	−5017	−4470	669	−4496	−3487	2791	−4191	1116	1394	−4156	−4228	−3615	−3972	−3687	−2499	1692	−2860	−2711	19900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
89(L)	−4414	−3800	−5638	−5628	−2290	−4980	−4628	−1886	−5423	3316	−1236	−5514	−4997	−4750	−5002	−5379	−4399	−2629	−3665	−3690	20000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
90(I)	1212	−1286	−3846	−3262	−1360	−3195	−2166	1616	−2918	1031	−493	−2824	−3211	−2583	−2782	−2308	1598	1299	−2020	−1668	20100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
91(P)	−1614	−2214	−3396	−3710	−4516	−2407	−3618	−4516	−3976	−4705	−3849	−2890	3993	−3625	−3900	666	−2068	−3354	−4610	−4474	20200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
92(D)	−4580	−4701	4174	−3014	−5700	−3967	−3905	−6376	−4478	−6024	−5744	−3355	−4501	−3870	−4926	−4440	−4750	−5894	−4922	−5231	20300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
93(E)	−1123	−2199	−983	2715	−2589	−2046	−942	−2250	−625	−2356	1979	−870	−2250	−554	−1093	463	932	−1902	−2660	−2064	20400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
94(H)	399	−1137	−2012	−14	1582	−2306	1600	246	−1252	190	−325	−1456	−2374	1474	−1479	−94	−905	896	−1557	−1158	20500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
95(Q)	−2742	−3142	−2766	−2681	−2790	−3344	−2460	−160	−1802	−2456	−2353	−2682	−3710	4317	−1866	−2894	−2844	−2559	−3295	−2711	20600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
96(A)	1981	−2315	−809	−268	−2645	−531	−579	−2374	232	−2350	−1445	−567	1217	711	445	447	−874	−1951	−2540	−1883	20700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
97(D)	491	−2351	1394	1381	−2671	−1854	1062	−2421	1010	−2367	−1440	−489	−1947	1017	362	−760	250	−623	−2535	−1852	20800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
98(V)	−2039	−1706	−4456	−3939	−1846	−3939	−3049	1986	−3656	1460	−826	804	−3870	−3351	−3565	−3105	−2000	2330	−2796	−2442	20900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
99(Y)	−4840	−3766	−5230	−5581	1898	−5109	−1300	−3727	−5135	−3041	−3132	−3723	−4964	−3861	−4501	−4357	−4690	−3883	3325	4377	21000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
100(E)	−163	−2353	−734	1681	−2674	−1859	888	−2422	1668	−792	−1443	777	−1952	890	286	−766	238	−1975	−2536	−1856	21100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−257	−8139	−2649	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

101(E)	1017	−2763	862	2042	−3060	−1913	−775	−2836	−495	−2773	−1886	1956	−2143	−136	−1056	265	−1185	−2377	−2948	−2207	21200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−9	−7891	−8933	−894	−1115	−338	−2261	*	*												
102(E)	−944	−2422	863	2138	−2740	−436	−567	−2493	894	−2437	−1515	−518	−1994	1767	−673	109	−885	−1023	−2605	−1917	21300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
103(I)	−2660	−2156	−5316	−4965	−2520	−5119	−4900	3165	−4894	297	−1251	−4775	−4828	−4705	−4975	−4470	−2642	2240	−4202	−3814	21400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
104(E)	1068	−2341	−760	2003	628	−1887	876	−2380	1240	−2347	−1436	−529	−1983	881	−618	−804	−855	−1954	−2530	−1862	21500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
105(P)	−343	−3144	1561	442	−3538	−489	−1216	−3329	−1038	−3274	−2420	−848	2974	−812	−1635	469	−1644	−2849	−3462	−2693	21600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
106(N)	−1173	−2375	−814	827	−2376	−2071	1767	−2279	−479	−2336	−1509	3151	−2218	−415	−957	−1093	−1120	−198	−2486	647	21700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
107(M)	−3415	−2890	−5826	−5252	−1352	−5488	−4282	1361	−5022	2621	2728	−5181	−4778	−4005	−4613	−4776	−3292	69	−3071	−3194	21800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
108(K)	−1941	−3098	−1997	−1232	−3650	−2740	−1025	−3210	3059	−3010	−2208	499	−2766	1457	1261	−1817	−90	−2858	−3002	−2622	21900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
109(P)	1129	−2426	−740	964	−2747	−1913	−589	−2491	1139	−2440	−1525	−552	1941	1446	−655	−480	−913	−2050	−2610	−1935	22000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
110(G)	−2276	−2907	−2347	−2709	−4832	3554	−3349	−5005	−3678	−5053	−4315	1193	−3507	−3243	−3937	−2418	−2674	−3974	−4703	−4521	22100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
111(A)	1730	−2349	958	−198	−2661	−1868	−535	−2405	927	−2362	−1444	414	−1966	788	−630	790	−840	−303	−2540	−1863	22200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
112(T)	1350	−1149	−14	−1461	−1155	−2314	−1111	758	−1275	−1024	1167	−1475	−2388	−1111	−1501	334	1843	354	−1581	−1182	22300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
113(L)	−3333	−2796	−5806	−5293	−1506	−5535	−4502	1096	−5103	2935	−282	−5232	−4857	−4172	−4762	−4864	−3236	506	−3264	−3351	22400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
114(A)	1769	−1525	158	−857	−1603	148	−891	−1181	−752	187	−712	−1040	−2228	660	−1135	−1111	−913	1305	−1913	−1442	22500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
115(F)	−4110	−3437	−5436	−5431	4216	−5143	−2159	−1742	−5074	563	−1124	−4290	−4871	−3987	−4561	−4547	−4016	−2374	−1356	−292	22600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
116(A)	3091	−1829	−3998	−4219	−4413	119	−3637	−4216	−4134	−4469	−3523	−2798	−2896	−3656	−3927	1514	−1679	−2983	−4632	−4539	22700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
117(H)	−5197	−4539	−4720	−5009	−4036	−4506	5435	−6314	−4911	−5786	−5667	−4954	−4960	−5011	−4732	−5391	−5395	−6022	−4063	−3641	22800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

118(G)	−4435	−4203	−5092	−5462	−5893	3834	−5028	−6627	−5765	−6297	−5970	−5141	−4804	−5546	−5385	−4727	−4815	−5862	−4924	−5849	22900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
119(F)	−4044	−3387	−5534	−5444	4093	−5246	−2370	−1514	−5107	1089	−868	−4443	−4880	−3998	−4592	−4639	−3934	−2200	−1536	−523	23000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
120(N)	885	−1899	−2020	−1781	−2956	−2135	−1925	−2602	−1809	3	−2052	3468	−2633	−1676	−2141	413	−1437	−2139	−3194	−2737	23100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
121(I)	−2673	−2169	−5324	−4969	−2477	−5123	−4876	3293	−4893	358	−1211	−4780	−4824	−4681	−4961	−4472	−2653	1969	−4158	−3791	23200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
122(H)	−3381	−3705	−3197	−3491	−4166	638	5216	−5496	−3798	−5304	−4811	−3481	−4185	−3770	−3879	−3508	−3702	−4793	−4170	−3759	23300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
123(Y)	−4816	−3757	−5210	−5549	3410	−5097	2153	−3719	−5105	−3041	−3127	−3715	−4955	−3851	−4483	−4344	−4669	−3870	−547	3677	23400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
124(G)	−1065	−2519	948	−272	−2820	1844	998	−2566	972	−284	−1622	1553	−2090	−229	−802	−938	−1011	−2133	−2708	−2021	23500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
125(Q)	−412	−2285	−2466	−2186	−2068	−2877	−2019	−1589	−1588	1526	−1121	−2187	−3153	3585	−1718	−2137	−1964	−1719	−2789	−2414	23600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
126(I)	−2254	−1916	−4813	−4439	−2466	−4221	−3932	3248	−4248	−1515	−1324	−4044	−4259	−4063	−4255	230	−2280	2003	−3673	−3237	23700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
127(K)	−888	−2234	334	1172	−2504	−1881	−546	−93	1370	−300	−1337	465	−1974	655	−646	−794	−827	1255	−2448	−1798	23800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
128(P)	715	−1925	−3618	−3897	−4464	653	−3594	−4274	−4053	−4520	−3596	−2770	3775	−3593	−3911	−1550	−1770	−3067	−4647	−4548	23900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
129(P)	479	−2398	−1173	−637	−2915	−2106	−848	−2610	−289	−2586	−1713	−884	2238	1247	2195	51	−1147	−2184	−2757	−2174	24000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
130(A)	1787	−2663	1377	529	−2976	−1992	−762	−2736	1785	−2680	−1776	−623	−2161	−319	−936	297	−1120	−2285	−2853	−2146	24100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−2336	−8139	−325	−894	−1115	−701	−1378	*	*												
131(F)	−1308	−1104	−2227	−2120	3516	−2093	−244	−196	−1891	64	66	−1626	−2278	−1503	−1798	−1617	−1350	−389	305	1335	24200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−38	−5840	−6882	−894	−1115	−3098	−179	*	*												
132(P)	−603	−937	−997	−1058	−1832	−1041	−1092	−1737	−1074	−1874	−1416	−992	3539	−1065	−1192	−789	−866	−1383	−1765	−1661	24300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−38	−5840	−6882	−894	−1115	−3098	−179	*	*												
133(K)	−804	−1483	−564	−230	−1920	−1335	−101	−1605	2889	−1630	−1021	−349	−1569	232	698	−786	−759	−1358	−1637	−1317	24400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−38	−5840	−6882	−894	−1115	−109	−3775	*	*												
134(D)	−2405	−4159	3349	−651	−4260	−261	−1744	−4307	−1947	−4207	−3514	2151	−2936	−1416	−2754	−2102	−2471	−3802	−4324	637	24500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

135(I)	−2047	−1713	−4504	−3983	−1821	−3943	−3061	2461	−3697	1581	−797	−3593	−3873	−3371	−3587	−342	−2009	1904	−2784	−2441	24600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
136(D)	−2024	−3444	3495	−680	−3868	−2331	−1596	−44	−1632	−3675	−2915	685	−2782	−1248	−2305	478	−2088	−3196	−3911	−3098	24700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
137(V)	−3122	−2888	−5092	−5160	−3522	−4180	−4687	−905	−5060	−2626	−2570	−4662	−4579	−4940	−4923	−4013	−3297	3796	−4414	−4190	24800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
138(I)	−53	−875	−3230	−2609	1867	−393	−1422	2613	−2236	−723	−81	−2157	−2608	−1885	−2086	−1633	276	−271	−1325	844	24900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
139(M)	315	−2345	−4754	−4279	−1396	−4001	−3301	−697	−3877	816	4676	−3879	−3994	−3361	−3676	−3242	−2531	−1114	−2746	−2629	25000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
140(V)	−2623	−2122	−5301	−4990	−2770	−5102	−5132	2415	−4945	−1532	−1474	−4791	−4869	−4890	−5102	−4483	−2619	3206	−4506	−3991	25100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
141(A)	3405	2528	−4529	−4796	−4340	−2257	−3851	−3901	−4447	−4351	−3532	−3057	−3052	−3976	−4112	−1643	−1844	−2929	−4572	−4519	25200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
142(P)	−4853	−4392	−5213	−5573	−5853	−4408	−5077	−6679	−5780	−6281	−6067	−5357	4310	−5648	−5396	−5166	−5194	−6092	−4900	−5786	25300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
143(K)	−4484	−4357	−4380	−3992	−5413	−4236	−3307	−5555	3994	−5171	−4707	−3921	−4535	−3079	−2169	−4529	−4408	−5264	−4403	−4729	25400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
144(G)	2167	−1833	−3963	−4199	−4430	2715	−3642	−4236	−4146	−4489	−3540	−2795	−2898	−3661	−3939	910	−1682	−2994	−4647	−4556	25500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
145(P)	−2604	−2948	−4094	−4235	−3544	−3269	−3767	−3353	−3912	−3066	2095	−3659	4036	−3912	−3822	−2883	−2963	−3249	−4027	−3787	25600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
146(G)	−4435	−4203	−5092	−5462	−5893	3834	−5028	−6627	−5765	−6297	−5970	−5141	−4804	−5546	−5385	−4727	−4815	−5862	−4924	−5849	25700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
147(H)	−2569	−3440	−1867	−1702	−3820	−2996	4731	−3830	634	−3639	−2963	−1838	1551	−1305	−748	−2470	−2510	−3482	−3434	−2990	25800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
148(T)	194	−1498	−3255	−2899	−2240	−2226	−2291	−1754	−2652	1634	−1430	−2330	−2747	−2399	−2684	567	2687	−1484	−2682	−2351	25900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
149(V)	−3122	−2888	−5092	−5160	−3522	−4180	−4687	−905	−5060	−2626	−2570	−4662	−4579	−4940	−4923	−4013	−3297	3796	−4414	−4190	26000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
150(R)	−4845	−4446	−5107	−4682	−5507	−4412	−3791	−5946	−2789	−5502	−5118	−4521	−4754	−3672	4219	−4989	−4832	−5644	−4538	−4993	26100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
151(R)	−962	−2395	−777	1012	−2721	76	1031	−2459	−142	−2413	−1501	−560	−2018	−128	2308	1224	−66	−2023	−2585	−1919	26200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

152(E)	−902	−2032	−899	2078	−2228	−1934	−611	−1897	−259	−221	−1156	520	−2024	816	−736	−858	1303	−287	−2295	537	26300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
153(Y)	−4820	−3765	−5219	−5565	3303	−5093	−1317	−3703	−5127	−3017	−3111	−3732	−4959	−3868	−4500	−4356	−4679	−3867	−565	4052	26400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
154(V)	129	−1901	−989	821	−2060	−1969	−654	−1704	498	−52	−1037	−703	−2057	695	−796	443	−344	1871	−2192	−1626	26500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
155(Q)	576	−2355	344	1156	−2675	−1856	−515	−508	1502	−2370	−1444	571	−1949	1878	419	−764	−822	−1976	−2538	−1856	26600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
156(G)	−3239	−3889	516	−2361	−5355	3646	−3337	−5629	−3818	−5498	−4951	−2619	−3905	−3187	−4377	−3211	−3532	−4837	−4895	−4826	26700%
—	−149	−500	232	44	−381	399	105	−627	211	−466	−721	277	393	45	95	359	117	−368	−295	−250	
—	−155	−3318	−9181	−2159	−366	−701	−1378	*	*												
157(G)	753	−2516	−789	488	−2848	2300	−672	−2582	596	−2529	−1627	481	−2112	−224	471	−962	−1024	−2149	−2694	−2033	27300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
158(G)	−52	−2212	−3792	−4133	−4698	3627	−3843	−4580	−4356	−4812	−3937	−3058	−3216	−3901	−4170	−1874	−2095	−3384	−4734	−4766	27400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
159(V)	−2485	−2030	−5123	−4769	−2667	−4797	−4593	2349	−4661	−1545	−1424	−4502	−4648	−4554	−4752	−4115	825	2986	−4159	−3678	27500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
160(P)	−2541	−3139	−2413	−2753	−4726	−2991	−3342	−5055	−3527	−5058	−4393	1199	4031	−3244	−3757	−2665	−2911	−4148	−4583	−4362	27600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
161(C)	1577	3078	1357	−656	−2664	−219	891	−2359	−617	−2434	−1576	−891	−2199	−545	−1093	872	−1043	−1946	−2701	−2102	27700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
162(L)	−2140	−2404	−3995	−3997	−2053	−3121	−3283	−1687	−3689	3041	−1200	−3360	−3626	−3433	−3567	480	−2414	−1973	−3145	−2755	27800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
163(I)	−2527	−2092	−5072	−4613	2047	−4674	−3911	2668	−4413	117	−841	−4323	−4439	−4023	−4320	−3916	−2488	2176	−3342	−3040	27900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
164(A)	3631	−2768	−4492	−4815	−4888	−2992	−4271	−4781	−4818	−5025	−4365	−3727	−3728	−4477	−4545	−2567	−2762	−3852	−4724	−4942	28000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
165(V)	−2623	−2122	−5301	−4990	−2770	−5102	−5132	2426	−4946	−1532	−1474	−4791	−4869	−4891	−5102	−4483	−2619	3200	−4506	−3991	28100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
166(H)	−495	−2631	903	−2051	722	−3242	3753	−2386	−2056	−2342	−1863	−2047	−3330	−1815	−2362	−2318	−2233	−2272	−981	3315	28200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
167(Q)	−4589	−4392	−3927	−4146	−5099	−4221	−4099	−5973	−3840	−5564	−5304	−4230	−4693	4575	−3826	−4704	−4772	−5612	−4577	−4751	28300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
168(D)	−2873	−4605	3943	−902	−4948	−2633	−2157	−5087	−2604	−4922	−4387	428	−3235	−1872	−3575	−2522	−3009	−4491	−4932	−3946	28400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

169(A)	1776	−1612	−1274	138	−1698	−2092	−816	−1295	150	−1526	−780	−943	1212	−538	−1013	−1056	−894	1275	−1968	635	28500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
170(S)	−1545	−2420	1001	−1518	−4049	−2206	−2206	−3839	−2264	−3938	−3103	−1627	−2814	−1909	−2758	2666	2313	−3045	−4143	−3560	28600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
171(G)	−2999	−3461	−2978	−3207	−5161	3669	−3454	−5283	15	−5188	−4565	−3174	−3946	−3312	−3140	−3128	−3317	−4492	−4622	−4749	28700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
172(N)	−887	−2349	−736	911	−2668	−1860	−518	−2415	711	−2363	−1440	1880	−1954	436	799	671	1230	−539	−2534	−1855	28800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
173(A)	3631	−2768	−4492	−4815	−4888	−2992	−4271	−4781	−4818	−5025	−4365	−3727	−3728	−4477	−4545	−2567	−2762	−3852	−4724	−4942	28900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
174(K)	−1368	−2597	−1358	−720	−2953	−2298	1933	−2603	1975	851	−1706	−958	−2360	859	1012	−1273	1094	−2245	−2690	−2165	29000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
175(D)	−27	−2613	2320	1049	−2923	−1962	1973	−2684	544	−2624	−1712	1408	−2115	345	−869	−969	−1058	−2231	−2793	−2087	29100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
176(V)	−1096	−938	−3279	−2658	−899	−2643	−1513	1265	1006	1388	−124	−2232	−2688	−1950	−2151	−1725	−311	1669	−1425	827	29200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
177(A)	3342	−1826	−4064	−4295	−4368	111	−3651	−4129	−4159	−4412	−3484	−2820	−2903	−3689	−3929	−1469	409	−2949	−4599	−4508	29300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
178(L)	−4414	−3800	−5638	−5628	−2290	−4980	−4628	−1886	−5423	3316	−1236	−5514	−4997	−4750	−5002	−5379	−4399	−2629	−3665	−3690	29400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
179(S)	2216	−1831	−3961	−4157	−4409	656	−3608	−4213	−4076	−4462	−3514	−2782	−2892	−3613	−3895	2686	−1675	−2982	−4623	−4523	29500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
180(Y)	−3634	−3050	−4918	−4872	36	−4597	−1405	223	−4437	−250	−1998	−3545	−4494	−3522	−4004	−3782	−3536	−2621	2928	4349	29600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
181(A)	3391	−1860	−3998	−4279	−4411	−2128	−3684	−4207	−4197	−4490	−3565	−2837	−2929	−3729	−3959	706	−1718	−3001	−4636	−4534	29700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
182(K)	370	307	113	−351	−2280	−1925	−615	324	1888	−2040	−1194	−632	−2022	−205	−736	1541	14	−1628	−2330	−1726	29800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
183(G)	2572	−2028	−3934	−4246	−4575	2752	−3783	−4406	−4316	−4661	−3751	−2958	−3070	−3837	−4092	−1679	−1898	−3191	−4701	−4686	29900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
184(I)	−2178	−1808	−4630	−4153	−2094	−4190	−3417	3121	−3909	311	−1023	698	−4099	−3656	−3864	−3388	−2148	1742	−3147	−2761	30000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
185(G)	−4435	−4203	−5092	−5462	−5893	3834	−5028	−6627	−5765	−6297	−5970	−5141	−4804	−5546	−5385	−4727	−4815	−5862	−4924	−5849	30100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

186(G)	1392	2751	−4353	−4536	−4308	2864	−3681	−4084	−4233	−4354	−3425	−2859	−2890	−3744	−3957	712	−1656	−2914	−4553	−4470	30200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
187(G)	855	−1822	−3738	−3769	−4188	2507	−3358	−3950	−3667	−4196	−3283	−2667	986	−3302	−3621	−1441	2334	−2867	−4408	−4236	30300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
188(R)	−3706	−3692	−4490	−3846	1391	−4057	−2273	−3795	−1906	−3355	−3181	−3458	−4263	−2675	3948	−3768	−3671	−3813	−2293	−1328	30400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−155	−8139	−3345	−894	−1115	−701	−1378	*	*												
189(A)	2844	−1670	−3814	−3873	−4048	−1958	−3316	−3787	−3686	−4061	−3156	−2598	−2734	−3301	−3572	1088	1907	−2713	−4286	−4136	30500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−7992	−9034	−894	−1115	−1303	−750	*	*												
190(G)	−4176	−3995	−4855	−5222	−5686	3828	−4823	−6386	−5533	−6087	−5741	−4896	−4606	−5312	−5178	−4461	−4560	−5613	−4754	−5635	30600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−7992	−9034	−894	−1115	−422	−1980	*	*												
191(V)	−2496	−2036	−5139	−4788	−2680	−4825	−4637	2522	−4686	−1549	−1431	−4527	−4668	−4584	−4783	−4148	629	2919	−4191	−3706	30700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
192(I)	−2760	−2307	−5270	−4785	1172	−4884	−3992	3346	−4576	662	−572	−4539	−4535	−4008	−4400	−4134	−2703	757	−3223	−3041	30800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
193(E)	454	−3086	−801	3279	−4125	−2346	−1868	−3919	−1868	−3932	−3156	−1291	1208	−1533	−2450	−1842	−2097	−3305	−4121	−3395	30900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
194(T)	−2738	−3109	−4509	−4810	−4918	−3305	−4346	−4865	−4769	−5072	−4551	−3987	−3998	−4580	−4545	−2999	4033	−4113	−4684	−4915	31000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
195(T)	−1323	−1975	−2766	−2978	−4207	−2152	−3105	−4004	−3295	−4229	−3354	134	−2901	−2988	−3387	−113	3742	−2969	−4405	−4119	31100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
196(F)	−4110	−3437	−5436	−5431	4216	−5143	−2159	−1742	−5074	563	−1124	−4290	−4871	−3987	−4561	−4547	−4016	−2374	−1356	−292	31200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
197(K)	−111	−2844	−1470	1220	−3294	−2415	−860	−2939	2448	−2798	−1945	−1056	−2475	767	2054	−1421	−1432	−2544	−2858	−2356	31300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
198(E)	545	−3735	1715	2880	−3981	−2308	−1442	−3818	−1408	−3725	−2924	−909	−2713	1281	−2087	−1777	−2041	−3331	−3909	−3046	31400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
199(E)	−4574	−4665	−2714	3919	−5655	−3995	−3886	−6219	−4238	−5898	−5604	−3415	−4513	−3838	−4570	−4456	−4726	−5786	−4878	−5197	31500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
200(T)	−1211	1331	−3446	−2962	−1399	−2495	−1930	−869	−2610	−1374	−769	−2403	−2815	−2309	−2526	−1685	3305	195	−1933	1430	31600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
201(E)	−1941	−3222	−921	3293	−3618	−2473	−1316	−3186	916	−3225	−2465	−1147	−2749	−923	−1064	−1790	−1902	−171	−3398	−2802	31700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
202(T)	−1286	−1898	−3764	−4016	−4329	−2157	−3572	−4120	−3959	−4408	−3517	−2789	−2947	−3588	−3797	697	3756	−2989	−4554	−4399	31800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

203(D)	−4580	−4701	4174	−3014	−5700	−3967	−3905	−6376	−4478	−6024	−5744	−3355	−4501	−3870	−4926	−4440	−4750	−5894	−4922	−5231	31900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
204(L)	−3705	−3122	−6060	−5527	−1359	−5814	−4569	1065	−5292	3069	−146	−5564	−4963	−4163	−4828	−5215	−3571	−1279	−3159	−3298	32000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
205(F)	−3777	−3220	−5271	−5259	4268	−4892	−2120	417	−4916	−1143	−1314	−4142	−4753	−3956	−4473	−4270	−3740	−1899	−1349	−269	32100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
206(G)	−4435	−4203	−5092	−5462	−5893	3834	−5028	−6627	−5765	−6297	−5970	−5141	−4804	−5546	−5385	−4727	−4815	−5862	−4924	−5849	32200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
207(E)	−4574	−4665	−2714	3919	−5655	−3995	−3886	−6219	−4238	−5898	−5604	−3415	−4513	−3838	−4570	−4456	−4726	−5786	−4878	−5197	32300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
208(Q)	−3157	−3746	−3170	−2450	−4497	−3515	−1763	−4161	−443	−3809	−3189	−2392	−3620	4200	1284	−3063	−2944	−3900	−3556	−3420	32400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
209(A)	2672	−1334	−3318	−2853	−1740	371	−2072	483	−2577	−1549	−928	−2359	−2798	−2295	−2567	−1629	191	932	−2245	−1899	32500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
210(V)	−2620	−2125	−5293	−4983	−2756	−5076	−5100	1877	−4932	−1522	−1466	−4777	−4855	−4870	−5082	−4456	−2619	3416	−4480	−3969	32600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
211(L)	−4414	−3800	−5638	−5628	−2290	−4980	−4628	−1886	−5423	3316	−1236	−5514	−4997	−4750	−5002	−5379	−4399	−2629	−3665	−3690	32700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
212(C)	−2243	5044	−4840	−4445	−1998	−3905	−3598	−31	−4138	449	−930	−3902	−4040	−3778	−4010	−3184	−2306	1347	−3209	−2883	32800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
213(G)	−4435	−4203	−5092	−5462	−5893	3834	−5028	−6627	−5765	−6297	−5970	−5141	−4804	−5546	−5385	−4727	−4815	−5862	−4924	−5849	32900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
214(G)	677	−2128	−3838	−4171	−4647	−3536	−3816	−4506	−4340	−4749	−3857	−3009	−3149	−3871	−4137	−1784	−2005	−3297	−4725	−4735	33000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
215(V)	378	724	−3707	−3104	−1180	−2986	−1919	1210	−2734	1302	−359	−2627	−3014	−2382	−2566	−2089	1123	1949	−1773	−1423	33100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
216(M)	−948	−1407	−1515	156	−1452	−2164	1677	−1030	−821	−1302	1976	−1113	−2245	−718	−1173	773	1715	1332	−1794	−1343	33200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
217(E)	1397	−2528	−725	2286	−2932	240	−791	−2681	328	−2645	−1744	−674	−2162	−351	−939	545	−1095	−2227	−2828	−2143	33300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
218(L)	−3705	−3122	−6060	−5527	−1359	−5814	−4569	1065	−5292	3069	−146	−5564	−4963	−4163	−4828	−5215	−3571	−1279	−3159	−3298	33400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
219(V)	−2600	−2108	−5251	−4894	−2568	−5025	−4783	2479	−4810	−1354	1358	−4683	−4772	−4654	−4895	−4362	−2584	3018	−4181	−3758	33500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

220(K)	−1633	−2905	−1573	706	−3375	−2487	−900	−3003	2925	−2849	−2008	−1128	−2541	1714	784	−1509	−105	−2617	−2894	−2418	33600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
221(A)	2352	2066	−2593	−2000	−947	−2434	−1271	−486	−32	−832	714	−1817	−2498	−1493	−1792	−1483	274	453	−1419	501	33700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
222(G)	224	−1905	−3562	−3696	−3684	3361	−3297	−3220	−3625	81	−2886	−2733	−2977	−3326	−3545	−1606	−1763	−2574	−4068	−3810	33800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
223(F)	−4781	−3756	−5207	−5542	4341	−5070	−1342	−3653	−5111	−2971	−3065	−3743	−4949	−3874	−4496	−4351	−4650	−3829	−591	1725	33900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
224(E)	−2413	−4114	221	3465	−4392	−2485	−1689	−4248	−1608	−4112	−3396	−1094	−2951	−1336	871	−2119	−2441	−3763	−4239	−3395	34000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
225(T)	−1461	−1864	−3139	−2645	−2659	−2483	−2136	−1734	−1646	−2359	−1761	−2298	−2936	−1995	920	−1748	3354	967	−2989	−2654	34100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
226(L)	−3831	−3266	−5314	−5148	−673	−5068	−2476	−1443	−4706	3059	−789	−4359	−4756	−3864	−4314	−4462	−3729	−2115	−1672	1736	34200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
227(V)	−1819	−1960	−4426	−4359	−2977	−3037	−3800	−439	−4098	−2145	−1909	−3451	−3600	−3879	−4011	−2397	2510	2999	−3974	−3608	34300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
228(E)	−2863	−4790	1397	3563	−4990	−2594	−2061	−5021	−2476	−4848	−4298	−1204	−3182	−1761	−3454	−2476	−2968	−4462	−4951	−3920	34400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
229(A)	2686	−1916	−1440	275	−2529	−292	−1240	−2176	−998	−2345	1183	−1184	−2355	−905	−1425	512	−1158	−1817	−2697	−2174	34500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
230(G)	−4435	−4203	−5092	−5462	−5893	3834	−5028	−6627	−5765	−6297	−5970	−5141	−4804	−5546	−5385	−4727	−4815	−5862	−4924	−5849	34600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
231(Y)	−4099	−3483	−4921	−5048	−109	−4705	−1565	−2914	−4494	−2334	2010	−3723	−4707	−3735	−4111	−4065	−4068	−3172	−847	4618	34700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
232(Q)	1711	−2410	−772	934	−2739	−1925	−604	−2477	−171	−2433	−1524	−574	−2035	2086	345	902	−923	−2042	−2608	−1941	34800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
233(P)	−3403	−4071	−1922	817	−5220	−3359	−3173	−5423	−3337	−5281	−4771	−2564	4045	−2989	−3760	−3320	−3624	−4817	−4763	−4636	34900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
234(E)	−2870	−4786	1265	3587	−4993	−2600	−2068	−5026	−2483	−4852	−4303	−1212	−3188	−1768	−3458	−2484	−2974	−4467	−4950	−3925	35000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
235(M)	−3089	−2618	−5526	−4976	−1443	−5128	−4045	653	−4735	1429	4269	−4803	−4610	−3915	−4423	−4378	−2995	1140	−3054	−3074	35100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
236(A)	3631	−2768	−4492	−4815	−4888	−2992	−4271	−4781	−4818	−5025	−4365	−3727	−3728	−4477	−4545	−2567	−2762	−3852	−4724	−4942	35200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

237(Y)	−4797	−3764	−5203	−5543	1114	−5069	−1339	−3694	−5111	−3013	−3107	−3741	−4951	−3876	−4497	−4354	−4666	−3859	−588	4723	35300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
238(F)	−3828	−3605	−4146	−4086	4292	−4207	−2060	−3492	774	−3071	−3005	−3556	−4434	−3287	−2952	−3868	−3858	−3571	−1593	−494	35400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
239(E)	−2775	−4471	−511	3582	−4815	−2610	−2057	−4863	−2317	−4711	−4124	1234	−3182	−1755	−3103	−2442	−2884	−4306	−4753	−3820	35500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
240(C)	−1407	5023	−4397	−4323	−3016	−2468	−3398	−1251	−3952	−2540	−2082	−3044	−3133	−3588	−3744	−1803	1473	1125	−3677	−3390	35600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
241(L)	−3370	−2847	−5795	−5233	−1386	−5465	−4298	708	−5010	2859	1349	−5155	−4777	−4026	−4622	−4755	−3254	814	−3103	−3213	35700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
242(H)	−2519	−4224	−445	946	−4287	−2505	4583	−4377	−1764	−4237	−3571	2007	−3009	−1439	−2361	−2209	−2569	−3893	−4267	−3353	35800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
243(E)	−3177	2571	−2701	3711	−4851	−3438	−3479	−4765	−3558	−4932	−4406	−3081	−4005	−3370	−3802	−3269	−3451	−4260	−4554	−4524	35900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
244(L)	−85	−1333	−3893	−3280	−1111	−3185	−2083	1066	−2910	2310	1961	−2823	−3170	−2501	−2721	−2289	−113	436	−1859	−1558	36000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
245(K)	−2513	−3173	−2941	−2370	−4402	−3094	−1824	−3895	3666	−3734	−3068	−2271	−3377	−1447	−616	−2537	751	−3485	−3625	−3471	36100%
—	−149	−500	233	43	−381	400	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−155	−3318	−9181	−196	−2974	−701	−1378	*	*												
246(L)	−3571	−3023	−5954	−5375	−1321	−5646	−4390	−632	−5138	2962	1671	−5358	−4852	−4044	−4689	−4963	−3436	742	−3082	−3239	36300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
247(I)	−2980	−2484	−5473	−5109	−1958	−5196	−4587	3728	−4915	267	−781	−4933	−4833	−4427	−4799	−4598	−2949	−64	−3627	−3397	36400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
248(V)	−1685	−1668	−4095	−3732	−2081	−3082	−2893	−227	−3402	−1488	1383	−3123	−3419	−3145	−3320	367	−1807	3332	−2874	−2504	36500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
249(D)	−2963	−4569	3864	−1039	−4953	−2751	−2187	−4998	767	−4822	−4260	−1424	−3314	−1891	−3072	−2624	−3060	−4467	−4770	−3962	36600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
250(L)	−2768	−2715	−4842	−4633	−1675	−3998	−3790	−1038	−4207	3056	−562	−4150	−4179	−3740	−3989	−3399	699	−1545	−3154	−3067	36700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
251(M)	−2822	−2356	−5342	−4861	−1759	−4985	−4151	2587	−4663	173	4005	−4649	−4601	−4076	−4487	−4251	−2764	766	−3321	−3210	36800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
252(Y)	−4562	−3630	−5142	−5401	1516	−4992	−1300	−3544	−4968	−2963	−2986	−3671	−4868	−3786	−4393	381	−4432	−3662	2413	4375	36900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
253(E)	−1959	−3457	−568	3135	−3841	−2347	−1410	−3622	−1165	−3547	−2751	−1000	−2716	1879	−1666	−1757	692	−3162	−3709	−2960	37000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

254(G)	−347	−2818	−1215	201	−3253	2635	−921	−2921	1474	−2822	−1972	−1002	−2459	−486	658	−1397	−1435	−2521	−2923	−2378	37100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
255(G)	−4435	−4203	−5092	−5462	−5893	3834	−5028	−6627	−5765	−6297	−5970	−5141	−4804	−5546	−5385	−4727	−4815	−5862	−4924	−5849	37200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
256(I)	−2042	−1769	−4321	−3740	−1316	−3753	−2668	3134	−3389	1017	1999	194	−3653	−2958	−3221	−2884	−1980	−344	−2325	−2073	37300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
257(A)	1914	−1640	−1237	128	−1748	−661	−793	−1355	−577	−49	−817	−905	−2155	−498	−993	1037	162	−1149	−2002	624	37400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
258(N)	365	−3809	1001	557	−4083	1196	−1518	−3930	−1535	−3838	−3055	3219	−2763	−1148	−2243	−1845	−2131	−3433	−4027	−3144	37500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
259(M)	−3656	−3159	−5816	−5350	−1349	−5421	−4248	−822	−4928	948	4920	−5248	−4838	−4039	−4539	−4860	−3558	−1557	−3044	−3030	37600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
260(R)	−1614	−1949	−2260	−1663	−886	−2765	−1089	−1596	−1089	360	−1133	2239	−2814	−1215	2408	−1789	−1535	−1468	1995	1546	37700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
261(Y)	−1548	−2973	568	−509	−2846	−2207	1172	−2986	441	−2941	−2110	1645	−2446	−659	−1264	−1389	−1509	−2582	−2924	3695	37800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
262(S)	279	−1844	−3877	−4131	−4448	136	−3634	−4260	−4132	−4511	−3561	−2782	−2903	−3648	−3936	3391	−1692	−3009	−4661	−4566	37900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
263(I)	−2653	−2149	−5311	−4961	−2538	−5114	−4905	2957	−4891	332	−1267	−4770	−4827	−4713	−4978	−4466	−2636	2521	−4218	−3821	38000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
264(S)	−2212	−2711	−4019	−4348	−4697	−2899	−4045	−4988	−4527	−5102	−4364	−3492	−3638	−4203	−4355	3681	−2664	−3902	−4616	−4605	38100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
265(N)	−2725	−4778	2906	990	−4896	−2486	−1922	−4885	−2320	−4718	−4140	3045	−3069	−1612	−3311	−2334	−2821	−4325	−4919	−3794	38200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
266(T)	−2061	−3396	−596	903	−4071	−2367	−1713	−3874	−1685	−3859	−3103	2125	−2850	−1369	−2277	−1897	3157	−3351	−4055	−3271	38300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
267(A)	3410	−2035	−3979	−4290	−4573	659	−3798	−4400	−4332	−4660	−3754	−2978	−3080	−3858	−4099	−1690	−1908	−3194	−4697	−4687	38400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
268(E)	−2118	−3486	−1036	2964	−3935	−2588	−1284	−3596	1878	−3417	−2636	−1209	−2827	1323	−773	−1923	−2032	−3199	−3455	−2917	38500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
269(Y)	−4524	−3618	−5100	−5310	1910	−4972	−1299	−3522	634	−2951	−2965	−3649	−4847	−3741	−4299	−4199	−4391	−3637	2997	4211	38600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−155	−8139	−3345	−894	−1115	−701	−1378	*	*												
270(G)	−4176	−3995	−4855	−5222	−5686	3828	−4823	−6386	−5533	−6087	−5741	−4896	−4606	−5312	−5178	−4461	−4560	−5613	−4754	−5635	38700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−7992	−9034	−894	−1115	−422	−1980	*	*												

TABLE Z-continued

271(D)	−2710	−4705	3025	1828	−4880	1758	−1932	−4863	−2320	−4703	−4115	−1084	−3073	−1621	−3297	−2330	−2809	−4301	−4894	−3793	38800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
272(Y)	−2497	−2175	−4651	−4137	2447	−4046	−2215	255	−3766	892	1558	−3537	−3886	−3109	−3468	−3181	−2410	−1282	−1615	3508	38900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
273(V)	−1425	−1250	−3480	−2894	−1283	−3035	−1955	691	−2570	−1	−429	−2568	−3060	942	−2514	−2129	1701	2578	−1907	−1553	39000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
274(T)	516	−1643	−1918	−1401	−2170	−2112	−1387	−1759	−1234	−2016	−1265	−1442	−2421	−1149	887	1341	2345	822	−2454	−2006	39100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
275(G)	677	−2128	−3838	−4171	−4647	3536	−3816	−4506	−4340	−4749	−3857	−3009	−3149	−3871	−4137	−1784	−2005	−3297	−4725	−4735	39200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
276(P)	−992	−2210	343	−359	−2447	−1960	−675	−2143	533	−2204	−1351	−651	2813	−260	−802	465	−939	−873	−2467	1093	39300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
277(R)	−1214	−2548	−1097	1072	175	−2145	−716	−2587	848	−2528	−1653	−795	−2228	−273	2862	417	−1133	−2191	−2671	−2084	39400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
278(V)	289	−2035	−5133	−4789	−2692	−4777	−4639	2142	−4689	−1561	−1443	−4511	−4649	−4585	−4784	−4102	−2487	3125	−4202	−3717	39500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
279(I)	−2265	−1919	−4828	−4452	−2473	−4254	−3954	3155	−4265	−1516	−1326	−4066	−4279	−4082	−4274	226	−2288	2182	−3688	−3250	39600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
280(D)	−1731	−3162	2329	−550	−3318	−2239	−1273	−3221	−1145	−3214	−2403	2295	−2573	−899	−1742	−1561	1851	−2804	−3366	1327	39700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
281(E)	1097	−2699	1227	2368	−2994	−2011	−796	−2753	381	−2704	−1808	−640	−2190	−358	−992	−1065	−1162	−2310	−2885	1152	39800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
282(E)	−166	−2372	859	1835	−2692	−1861	1182	−2444	490	−2388	−1462	590	478	1356	−620	116	−837	−1994	−2555	−1871	39900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
283(T)	228	−1688	−3655	−3444	−3179	−2145	−2891	−2611	−3215	−3076	−2328	−2557	−2821	−2916	−3214	2251	2366	1397	−3549	−3270	40000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
284(K)	−2991	−3623	−3848	−2331	−4472	−3512	−1356	−3763	2942	−3413	1860	−2188	−3430	−937	2705	−2874	−2650	−3556	−3267	−3189	40100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
285(E)	443	−2370	732	1690	−2691	−1863	−526	−2442	1639	−2385	−1460	−498	−1960	1120	437	106	−836	−1992	−2552	−1869	40200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
286(A)	1871	−2286	−843	814	−2570	−1928	−578	269	1096	−2279	−1391	−586	−2019	−136	1056	−62	−881	−1889	−2488	−1849	40300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
287(M)	−3656	−3159	−5816	−5350	−1349	−5421	−4248	−822	−4928	948	4920	−5248	−4838	−4039	−4539	−4860	−3558	−1557	−3044	−3030	40400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

288(K)	−1646	−2891	−1591	287	−3346	−526	−912	−2971	2831	−2832	−1997	−1146	−2554	−472	1762	−1527	−1524	−2597	−2885	1245	40500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
289(E)	−172	−2394	367	2205	−2713	−487	−545	−2465	−134	−2409	−1485	1305	−1975	663	831	−795	72	−2015	−2577	−1891	40600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
290(C)	1574	3024	−4584	−4122	−2155	−3932	−3330	1746	−3870	−1406	−1109	−3691	−3957	−3613	−3805	−3144	−2046	2342	−3118	−2720	40700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
291(L)	−187	−2175	−4307	−3889	−898	−3779	−2344	−944	−3485	2855	−476	−3390	−3782	−3025	−3298	−2972	−2345	−1269	−1846	1565	40800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
292(K)	862	−2347	143	1211	−2665	−1855	873	−2414	1692	−2362	917	−492	−1949	889	−603	−763	783	−1968	−2532	−1851	40900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
293(D)	−2148	−3878	2790	1765	−4119	−2356	−1511	−3962	−1467	−3852	−3075	24	−2782	−1139	2142	−1879	−2163	−3473	−4024	−3155	41000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
294(I)	−2630	−2131	−5302	−4991	−2737	−5092	−5106	3464	−4941	−1495	−1447	−4789	−4862	−4869	−5086	−4473	−2627	2071	−4467	−3968	41100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
295(Q)	346	−3134	−1818	−1401	−3862	−2760	−1314	−3433	1329	−3271	−2513	−1545	−2936	3817	−430	−2018	−2031	−3060	−3278	−2908	41200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
296(S)	−1354	−2895	1712	354	−3192	−2068	−914	−2967	−621	−2903	−2012	1817	−2288	724	−1177	1978	−96	−2508	−3076	−2340	41300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
297(G)	−4435	−4203	−5092	−5462	−5893	3834	−5028	−6627	−5765	−6297	−5970	−5141	−4804	−5546	−5385	−4727	−4815	−5862	−4924	−5849	41400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
298(E)	−437	−2374	−769	2013	−2697	−1895	−552	−2438	623	−2389	−1472	−536	−1991	−97	777	829	1488	−1999	−2559	−1890	41500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
299(F)	−4347	−3577	−4619	543	3858	−4820	−1320	−3438	−4609	−2900	−2894	−3532	−4750	−3628	−4209	−4078	−4237	−3546	−601	2917	41600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
300(A)	2827	−1603	−4068	−3628	−2047	−3165	−2823	1205	−3349	−1486	−1089	−3103	−3432	−3073	−3298	−2387	197	1100	−2812	−2447	41700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
301(K)	−2364	−3363	−2464	163	−4038	−3041	−1188	−3501	2928	−3234	−2487	−1693	−3047	−758	2488	−458	−2152	−3194	−3159	−2895	41800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
302(M)	−893	−2361	740	1780	−2680	−537	−524	−2429	930	−2376	1895	−498	−1958	−66	722	775	−833	−1982	−2545	−1864	41900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
303(W)	−2965	−2553	−4795	−4482	3045	−4315	−1779	−1426	−4093	92	−987	−3564	−4179	−3330	−3743	−3475	−2878	664	4754	−84	42000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
304(I)	−2621	−2136	−5246	−4859	−2364	−4987	−4577	3052	−4747	−250	1043	−4641	−4713	−4488	−4769	−4301	−2597	2384	−3934	−3596	42100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

305(L)	684	−1319	−1741	761	−1375	−2223	−1037	−927	−1042	1693	−533	−1287	−2325	−915	−1349	767	−152	84	−1756	−1329	42200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
306(E)	364	−4165	621	3314	−4393	−2398	−1686	−4282	−1836	−4169	−3446	728	−2893	−1339	−2626	−2045	−2402	−3767	−4362	−3401	42300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
307(N)	−939	979	−1235	−681	−1738	1352	935	−1357	−549	−1572	−816	2186	−2155	−482	165	−1022	−880	30	−1990	1446	42400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
308(Q)	667	−2393	−773	511	−2721	−316	−544	−2465	1211	−2405	−1484	584	−1989	2135	1528	−812	−868	−2019	−2566	−1895	42500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
309(A)	2050	−1857	−1081	−526	−2013	−2012	188	−1645	−385	−1213	−1011	486	−2103	−346	1657	−953	306	−261	−2169	−1624	42600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
310(G)	−1848	−2469	−2089	−2292	1262	2944	−2358	−3563	−2904	−3628	−3017	2347	−3167	−2550	−3185	−1986	−2162	−3006	−2814	−1874	42700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
311(Y)	475	1019	−1606	−1042	225	−2192	−935	−946	−891	−1226	1222	357	−2267	1002	1577	−1172	−888	−800	−1730	2446	42800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
312(P)	−87	−2372	−1362	756	−3738	−2205	−2007	−3445	−1924	−3575	−2761	−1555	3598	−1697	−2362	−1566	286	−2803	−3832	−3277	42900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−2336	−8139	−325	−894	−1115	−701	−1378	*	*												
313(K)	−804	−1483	−564	−230	−1920	−1335	−101	−1605	2889	−1630	−1021	−349	−1569	232	698	−786	−759	−1358	−1637	−1317	43000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−38	−5840	−6882	−894	−1115	−3098	−179	*	*												
314(E)	−766	−1695	521	2831	−2050	−1029	−293	−1804	−118	−1919	−1331	69	−1441	−4	−527	−653	−814	−1512	−1988	−1505	43100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−38	−5840	−6882	−894	−1115	−109	−3775	*	*												
315(T)	−942	−2382	−739	1086	−2714	151	−581	−2459	−171	−2415	−1499	414	−2004	−128	839	1365	1730	−2017	−2592	−1915	43200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
316(M)	−2196	−1920	−4499	−3891	1726	−3822	−2504	−645	−3523	1973	3030	−3442	−3673	−2938	−3257	−2944	−2114	326	−2014	1662	43300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
317(H)	−883	−2314	−747	517	−2618	−1863	1714	−647	1272	−2322	−1408	1011	472	−69	433	−772	1411	−299	−2507	−1836	43400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
318(A)	2474	−2397	−816	−367	−2797	−273	−722	−2529	555	−2507	−1610	592	−2110	837	−805	−138	−1006	−2092	−2699	−2039	43500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
319(M)	−154	−986	−2485	−337	1024	−375	−1232	325	−444	867	1235	−1752	−2474	−1419	1020	−1455	670	−411	831	535	43600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
320(R)	−1311	−2432	−1349	−724	−2724	−2272	−799	−2361	613	−644	1079	976	597	−382	2908	−1246	−1219	−2044	−2579	−2061	43700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
321(R)	897	−2364	−833	905	−2678	−1930	−568	−2405	1293	−2366	1485	−575	−2020	−177	2045	95	−893	−1984	−2543	−1892	43800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

322(N)	505	−2300	−750	523	−2598	121	−525	−594	485	95	−1395	1720	−1957	348	224	551	−821	−1910	−2497	−1828	43900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
323(E)	444	−2266	−766	1488	−2551	−1871	−533	−2276	889	−2266	1474	1478	−1963	682	−629	−781	12	−279	−2472	476	44000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
324(N)	1511	−1770	−728	−204	−2244	−1781	−426	282	601	−2121	−1133	1769	−1827	1398	447	−748	−766	−1713	−1779	−1595	44100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
325(N)	1053	−3109	1756	1735	−3404	−2143	−1074	−3191	−846	−3124	−2254	2158	−2417	−657	−1430	−1361	197	−2727	−3303	−2540	44200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
326(H)	−2064	−3071	−1245	−1267	−3262	−2570	4711	−3611	−1060	−3528	−2812	−1479	−2961	1288	−1287	670	−2133	−3161	−3310	−2601	44300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
327(Q)	−891	−2294	332	948	−2585	−739	−537	−2316	−131	−138	−1391	−518	1404	2021	−634	−785	619	−561	−2495	−1830	44400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
328(I)	−1632	−1661	−2846	−86	−1626	−2983	−1663	3240	−1327	−191	−722	−2160	−3016	−1615	822	−2097	−1556	−560	−2157	−1805	44500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
329(E)	−2734	−3605	−1382	3593	−3624	−2986	−2317	−3317	−2175	−3167	1983	−1898	−3440	−2054	−2556	−2649	−2820	−3234	−3920	−3370	44600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−2336	−8139	−325	−894	−1115	−701	−1378	*	*												
330(W)	−1530	−1265	−2068	−1964	479	−1810	−483	−1181	−1470	−968	−802	−1648	−2104	−1454	−1405	−1757	−1583	−1218	5462	838	44700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−38	−5840	−6882	−894	−1115	−109	−3775	*	*												
331(K)	8	−2067	−905	437	−2275	−1941	−611	−307	2031	−2031	−1189	−636	−2030	1425	−709	−864	−350	1337	−2323	−1722	44800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
332(V)	−2445	−2012	−5067	−4708	−2628	−4682	−4459	1586	−4580	−1533	−1402	−4414	−4575	−4456	−4653	−3990	1117	3227	−4065	−3597	44900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
333(G)	−4435	−4203	−5092	−5462	−5893	3834	−5028	−6627	−5765	−6297	−5970	−5141	−4804	−5546	−5385	−4727	−4815	−5862	−4924	−5849	45000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
334(E)	1477	−2744	−762	2410	−3114	−2113	−884	−2850	−445	−2792	−1912	619	−2297	−451	1346	−1191	−1280	−2412	−2952	−2288	45100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
335(K)	−1204	−2643	366	1309	−2998	−2086	−724	−2722	2626	−2637	−1741	−718	−2200	1198	862	−1073	−1133	−2287	−2770	−2133	45200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
336(L)	−3571	−3023	−5954	−5375	−1321	−5646	−4390	−632	−5138	2962	1671	−5358	−4852	−4044	−4689	−4963	−3436	742	−3082	−3239	45300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
337(R)	−4845	−4446	−5107	−4682	−5507	−4412	−3791	−5946	−2789	−5502	−5118	−4521	−4754	−3672	4219	−4989	−4832	−5644	−4538	−4993	45400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
338(E)	943	−2422	1002	1200	−2741	377	−572	−2493	982	−2439	−1517	−522	−1998	−117	−681	1129	−534	−2044	−2609	−1921	45500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

339(M)	−3391	−2886	−5774	−5202	−1338	−5407	−4210	−576	−4943	1721	4369	−5109	−4742	−3963	−4548	−4689	−3273	771	−3038	−3137	45600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
340(M)	−1812	−1601	−3997	−3375	696	−3401	−2222	−516	−2983	441	4360	−2991	−3334	−2559	641	−2503	−1740	404	−1934	−1661	45700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8139	−9181	−894	−1115	−701	−1378	*	*												
341(P)	102	−1789	−1729	−1313	428	−2112	−1410	−2021	−1236	−2235	−1470	−1394	3205	695	−1607	703	−1207	−1700	−2613	−2121	45800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−259	−8139	−2637	−894	−1115	−701	−1378	*	*												
342(W)	−3486	−3022	−4312	−4121	1891	−4228	−1173	−2749	310	−2389	−2250	−3147	−4206	−2971	−3005	−3484	−3396	−2818	5644	611	45900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−239	−7889	−2749	−894	−1115	−1590	−583	*	*												
343(I)	−2220	−1737	−4860	−4531	−2271	−4617	−4497	3348	−4448	−1059	−1008	−4311	−4407	−4340	−4559	−3974	−2216	2150	−3915	−3445	46000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−114	−7660	−3820	−894	−1115	−1149	−865	*	*												
344(A)	1699	−2218	532	−33	−2555	−553	−413	−2304	1212	−2260	−1348	582	−1822	37	−536	966	−724	−1859	−2439	−1755	46100%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−10	−7753	−8795	−894	−1115	−897	−1111	*	*												
345(A)	1523	−2068	−769	−231	−2383	1040	−522	−2084	928	−2120	−1245	−517	−1922	1335	−611	251	−768	−123	−2361	−1732	46200%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−9	−7949	−8991	−894	−1115	−1432	−668	*	*												
346(N)	−1650	−3264	1760	−348	−3555	−230	−1131	−3362	324	−3285	−2448	2847	1624	−733	−1607	−1438	−1645	−2887	−3466	−2662	46300%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−193	−7842	−3049	−894	−1115	−1432	−668	*	*												
347(K)	150	−2932	−2433	−1483	−3710	−2747	−907	−3141	3369	−2897	−2178	−1487	−2774	−488	1175	−1994	−1888	−2848	−2822	−2610	46400%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−11	−7660	−8702	−894	−1115	−1824	−479	*	*												
348(L)	−740	−922	−1768	154	−921	−2070	−829	1384	247	1472	−100	−1202	−2134	−805	485	−1085	−677	471	−1340	−944	46500%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−11	−7660	−8702	−894	−1115	−943	−1059	*	*												
349(V)	138	−1046	−3186	−2599	−1089	−2803	−1711	589	645	945	−236	−2305	−2830	−2012	−2236	−1892	−1154	2537	−1701	−1344	46600%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−9	−7842	−8885	−894	−1115	−380	−2109	*	*												
350(D)	−2086	−3722	2888	1158	−4001	1601	−1510	−3811	−1573	−3773	−3018	−904	−2733	−1154	−2294	−1833	−2125	575	−3993	−3117	46700%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8046	−9088	−894	−1115	−701	−1378	*	*												
351(K)	−78	−2308	398	−137	−2626	−483	542	−2374	2441	−2323	−1400	−447	531	720	−562	−724	290	−1929	−2493	−1812	46800%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−8	−8046	−9088	−894	−1115	−701	−1378	*	*												
352(D)	898	−1911	1604	−335	−2089	−1892	−573	−1746	489	−144	811	−609	34	−182	−708	541	−74	−281	−2188	−1605	46900%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−10	−7745	−8787	−894	−1115	−701	−1378	*	*												
353(K)	−1676	−2544	−1956	−1135	−2823	−2531	−800	−2469	2955	−2426	−1683	−1240	−2548	−407	1469	−1628	−1517	−119	−2497	1619	47000%
—	−149	−500	233	43	−381	399	106	−626	210	−466	−720	275	394	45	96	359	117	−369	−294	−249	
—	−11	−7649	−8691	−894	−1115	−701	−1378	*	*												

TABLE Z-continued

354(N)	−1074	−1925	−1092	−1190	−3523	−1781	−1760	−3345	−1701	−3432	−2589	3429	1327	−1460	−2119	1182	−1362	−2558	−3601	−3046	47100%
—	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
—	*	*	*	*	*	*	*	*	0												

HMMER2.0 [2.2 g]	File format version: a unique identifier for this save file format.
NAME Functionally Verified KARIs	Name of the profile HMM
LENG 354	Model length: the number of match states in the model.
ALPH Amino	Symbol alphbet: This determines the symbol alphabet and the size of the symbol emission probability distributions. IAmino, the alphabet size is set to 20 and the symbol alphabet to "ACDEFGHIKLMNPQRSTVWY" (alphabetic order).
MAP yes	Map annotation flag: If set to yes, each line of data for the match state/consensus column in the main section of the file is followed by an extra number. This number gives the index of the alignment column that the match state was made from. This information provides a "map" of the match states (1 . . . M) onto the columns of the alignment (1.alen). It is used for quickly aligning the model back to the original alignment, e.g. when using hmmalign-mapali.
COM hmmbuild-n Functionally Verified KARIs exp-KARI.hmm exp-KARI_mod.aln	Command line for every HMMER command that modifies the save file: This one means that hmmbuild (default patrameters) was applied to generate the save file.
COM hmmcalibrate exp-KARI.hmm	Command line for every HMMER command that modifies the save file: This one means that hmmcalibrate (default parametrs) was applied to the save profile.
NSEQ 25	Sequence number: the number of sequences the HMM was trained on
DATE Mon Dec 8 17:34:51 2008	Creation date: When was the save file was generated.
XT −8455 −4 −1000 −1000 −8455 −4 −8455 −4	Eight "special" transitions for controlling parts of the algorithm-specific parts of the Plan7 model. The null probability used to convert these back to model probabilities is 1.0. The order of the eight fields is N->B, N->N, E->C, E->J, C->T, C->C, J->B, J->J.
NULT −4 −8455	The transition probability distribution for the null model (single G state).
NULE 595 −1558 85 338 −294 453 −1158 197 249 902 −1085 −142 −21 −313 45 531 201 384 −1998 −644	The extreme value distribution parameters μ and lambda respectively; both floating point values. These values are set when the model is calibrated with hmmcalibrate. They are used to determine E-values of bit scores.
EVD −333.712708 0.110102	

SEQUENCE LISTING

The patent contains a lengthy "Sequence Listing" section. A copy of the "Sequence Listing" is available in electronic form from the USPTO web site (http://seqdata.uspto.gov/?pageRequest=docDetail&DocID=US09422582B2). An electronic copy of the "Sequence Listing" will also be available from the USPTO upon request and payment of the fee set forth in 37 CFR 1.19(b)(3).

What is claimed is:

1. A method for producing isobutanol comprising:

a. providing a recombinant yeast cell comprising:

(i) an engineered isobutanol production pathway comprising a heterologous polypeptide having ketol-acid reductoisomerase (KARI) activity, wherein said polypeptide having KARI activity has at least 99% identity to the amino acid sequence set forth in SEQ ID NO: 419;

(ii) a modification that eliminates expression or activity of a pyruvate decarboxylase polypeptide, wherein said modification is a deletion of an endogenous gene encoding said pyruvate decarboxylase polypeptide;

(iii) a modification that eliminates expression or activity of an NAD-dependent glycerol-3-phosphate dehydrogenase polypeptide, wherein said modification is a deletion of an endogenous gene encoding said NAD-dependent glycerol-3-phosphate dehydrogenase polypeptide; and

(iv) a modification that eliminates expression or activity of a polypeptide affecting Fe—S cluster biosynthesis in the yeast, wherein said modification is a deletion of an endogenous gene encoding said polypeptide affecting Fe—S cluster biosynthesis in yeast; and

b. contacting said recombinant yeast cell of a) with a carbon substrate under conditions whereby isobutanol is produced; wherein at least a portion of said contacting occurs under anaerobic conditions.

2. The method of claim **1**, wherein said recombinant yeast cell comprises a cytosol-localized acetolactate synthase.

3. The method of claim **1**, wherein the isobutanol is removed as it is produced.

4. The method of claim **3**, wherein said recombinant yeast cell is contacted with an organic extractant.

5. The method of claim **4**, wherein said organic extractant comprises C_{12} to C_{22} fatty acids.

6. The method of claim **1**, wherein the molar ratio of isobutanol to glycerol produced is greater than 1.

7. The method of claim **1**, wherein said recombinant yeast cell further comprises a modification that eliminates expression or activity of an acetolactate reductase polypeptide in the yeast, wherein said modification is a deletion of an endogenous gene encoding said acetolactate reductase polypeptide.

8. The method of claim **1**, wherein said recombinant yeast cell further comprises a modification that eliminates expression or activity of an aldehyde dehydrogenase polypeptide in the yeast, wherein said modification is a deletion of an endogenous gene encoding said aldehyde dehydrogenase polypeptide.

9. A method for producing isobutanol comprising:

a. providing a recombinant yeast cell comprising:

(i) an engineered isobutanol production pathway comprising a heterologous polypeptide having ketol-acid reductoisomerase (KARI) activity, wherein said polypeptide comprises the amino acid sequence set forth in SEQ ID NO 419;

(ii) a modification that eliminates expression or activity of a pyruvate decarboxylase polypeptide, wherein said modification is a deletion of an endogenous gene encoding said pyruvate decarboxylase polypeptide;

(iii) a modification that eliminates expression or activity of an NAD-dependent glycerol-3-phosphate dehydrogenase polypeptide, wherein said modification is a deletion of an endogenous gene encoding said NAD-dependent glycerol-3-phosphate dehydrogenase polypeptide; and

(iv) a modification that eliminates expression or activity of a polypeptide affecting Fe—S cluster biosynthesis in the yeast, wherein said modification is a deletion of an endogenous gene encoding said polypeptide affecting Fe—S cluster biosynthesis in yeast; and

b. contacting said recombinant yeast cell of a) with a carbon substrate under conditions whereby isobutanol is produced; wherein at least a portion of the said contacting occurs under anaerobic conditions.

10. The method of claim **9**, wherein said recombinant yeast cell further comprises a modification that eliminates expression or activity of an acetolactate reductase polypeptide in the yeast, wherein said modification is a deletion of an endogenous gene encoding said acetolactate reductase polypeptide.

11. The method of claim **9**, wherein said recombinant yeast cell further comprises a modification that eliminates expression or activity of an aldehyde dehydrogenase polypeptide in the yeast, wherein said modification is a deletion of an endogenous gene encoding said aldehyde dehydrogenase polypeptide.

12. The method of claim **9**, wherein said recombinant yeast cell comprises a cytosol-localized acetolactate synthase.

13. The method of claim **9**, wherein the isobutanol is removed as it is produced.

14. The method of claim **13**, wherein said recombinant yeast cell is contacted with an organic extractant.

15. The method of claim **14**, wherein said organic extractant comprises C_{12} to C_{22} fatty acids.

16. The method of claim **9**, wherein the molar ratio of isobutanol to glycerol produced is greater than 1.

* * * * *